Antigonadotropic activity of hop extract

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Biological activity of water soluble fractions F-1 and F-2, which were extracted from hop, was studied and its action mechanism was speculated using the immature female SD rats. Administration of the substance significantly inhibited the effects of pregnant mare serum gonadotropin (PMSG) on 22-day-old female rats. Thus, PMSG-induced increases in ovarian weight, estrogen secretion, number of ovulated egg, progesterone production, uterine thymidine kinase activity, and plasma LH level were suppressed significantly. Furthermore, addition of the substance to incubated ovarian cells of the second day after PMSG injection resulted in suppression of FSH-induced estradiol secretion in vitro, probably via cAMP-dependent mechanism. But addition of the substance to incubated pituitary cells from ovariecetomized rats did not change in LH secretion.

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Although estrogenic activity in the lipophilic fraction of hop extracts has been studied by several investigators (1-4), none of these studies has yielded conclusive results. We have previously reported that de-fatted, water-soluble extracts from hops prevent a PMSG-induced increase in ovarian weight in immature rats (5). The aim of the present in vivo and in vitro experiments is to study the effects of hop extracts on the stimulatory action of exogenous gonadotropin (PMSG), using as our parameters LH release, production of estradiol (E2) and progesterone (P), ovulation, and uterine thymidine kinase (TK) activity in immature rats. We have also tried to determine whether the hop extracts act directly on the pituitary. The data obtained suggest that these extracts inhibit the response to PMSG in immature rats.

Materials and methods

Preparation of hop extracts

Details of the methods for preparation and physico-chemical analysis of the hop extracts have been described in our previous report (5). To summarize, hop (humulus lupulus) granules were de-fatted and dried to powder with acetone. Extraction of the powder was performed with borate buffer, pH 10.0, and the filtrate was adjusted to pH 4.0 with HCl. Then the precipitate, collected by centrifugation, was dissolved in distilled water and was adjusted to pH 9.0 with NaOH. Two active fractions, designated F-1 and F-2 respectively, were obtained from the dialysate of the above solution by multi-step column chromatography using DEAE-cellulose, Sephadex G-100, and DEAE-cellulofine. As mentioned in our previous report (5), the apparent molecular weights of the partially purified fractions thus obtained were nearly 80,000 (F-1) and 66,000-80,000 (F-2). These fractions were water-soluble, and acidic and neutral sugars were detected in the hydrolysate.

Animals

Female Sprague-Dawley rats were used throughout the experiments. The rats were housed in a temperature- and humidity-controlled room with an automatic 14-h light and 10-h dark cycle (light cycle: 06.00 to 20.00). Food and water were provided ad libitum. The experiments were performed on 22-day-old rats which, at precisely 10.00, were subcutaneously injected with 25 IU/0.2 ml of PMSG in saline solution through the experiment unless mentioned otherwise. We have previously reported that PMSG induces an LH surge between 20.00 and 23.00 on the second day, ovulation takes place in the morning on the third day, and P release from the luteal cells reaches maximum levels on the sixth day after the administration of PMSG (6). In the experiment of ovulation, 8 IU/l of PMSG was used following the procedure by Herlitz et al. (7). At autopsy, the weights of the whole body, uterus and ovaries were measured.

In vivo experiments

Vehicle (0.2 ml of saline) or fractions from hop extract dissolved in 0.2 ml of saline were injected subcutaneously into the PMSG-primed rats twice a day (at 10.00 and 17.00) for 2, 3 or 4 days. The first injection was performed 30 min after the PMSG injection. PMSG (1000 IU/A Lot. YO 12) was purchased from Teikoku Hormone Mfg. Co, Ltd.
(a) **Serum LH levels.** Vehicle or hop fractions were administered to six rats respectively for three days. Both F-1 and F-2 fractions were administered using two different dosage regimens, such that the total dosage per rat was either 0.5 mg or 5.0 mg. Blood was collected by cardiac puncture under ether anesthesia at 10.00 on the fourth day after PMSG injection; the samples were placed at room temperature for 20 min and centrifuged at 1,000 x g for 15 min at 15°C to obtain the serum. LH in the serum was assayed by radioimmunoassay (RIA). NIAMDD-Rat LH-15 (FSH contamination: 0.04 x NIH-FSH-S1) was radiiodinated by lactoperoxidase (Boehringer Mannheim, Lot. 1270316). NIAMDD-Rat LH-RP-1 (FSH contamination: 0.54 x NIH-FSH-S1) and NIAMDD-Anti-Rat LH serum-S5 were used for RIA. The standard curve ranged from 20 μg/l to 1,000 μg/l. Intra-assay and interassay coefficients of variation were 9.7% (N = 10) and 9.2% (N = 3).

(b) **Estradiol (E₂) levels.** We chose to measure E₂ production in the culture medium of ovarian cells isolated from rats which had received injections of hop extract in vivo. Ovarian cells were prepared from ovaries 48 h after PMSG injection, following the procedure described by Hamberger et al. (12). Vehicle or hop fractions were administered to five rats respectively for two days. Both F-1 and F-2 fractions were administered using two different dosage regimens, such that the total dosage per rat was either 0.5 mg or 5.0 mg. After rinsing in Hanks’ solution, 10⁶ ovarian cells were suspended in 1 ml Eagle’s MEM and incubated at 37°C for 18 h in an atmosphere of 95% O₂-5% CO₂. The incubation medium was centrifuged at 600 × g at 4°C for 10 min, and the supernatant was stored at —30°C until E₂ assays were performed. E₂ levels were assayed by RIA using a kit manufactured by the Daiichi-Radioisotope Co. Ltd. The standard curve ranged from 1.6 ng/l to 400 ng/l. The E₂ values were expressed as ng per pair of ovaries. Intra-assay and interassay coefficients of variation were 5.3% (N = 8) and 7.2% (N = 3), respectively.

(c) **Progesterone (P) levels.** As above, we chose to measure P production in the culture medium of luteal cells isolated from rats which had received injections of hop extract in vivo. Vehicle or hop fractions were administered to six rats respectively for four days. Both F-1 and F-2 fractions were administered using the same dosage regimen, such that the total dosage per rat was 5.0 mg. Luteal cells were isolated from the ovaries six days after PMSG injection, following a procedure which combines collagenase digestion with a density gradient method described by us in a previous paper (6). After isolation, 10⁶ luteal cells were incubated in 1 ml Eagle’s MEM containing 10% fetal calf serum (Gibco, Lot. 44D-0505) at 37°C for 18 h in an atmosphere of 95% O₂-5% CO₂, with or without 10⁻³ or 10⁻¹ IU/l of human chorionic gonadotropin (hCG: Sigma, CG-B 3360 IU/mg, Lot. 32F-0023). The medium was stored at —30°C until P assay was performed. P levels were assayed by RIA following the method of Lindner (8) and Makino (9). The P values were expressed as ng per 10⁵ cells. The standard curve ranged from 20 pg to 1,000 pg per tube. Intra-assay and interassay coefficients of variation were 9.7% (N = 10) and 12.7% (N = 3), respectively.

(d) **Thymidine kinase (TK) activity in the uterine tissue.** Fifteen rats were pretreated with 10 IU/l of PMSG at 10.00. Vehicle or hop fractions were administered to three groups of five each at 10.30 and 17.00, and at 10.00 of the next day. Both F-1 and F-2 fractions were given using two different dosage regimens, such that the total dosage per rat was either 0.5 mg or 5.0 mg. The uterus were removed at 15.30, i.e. 6 h after the last injection, and stored at —80°C until TK assay was performed. The TK activity per mg of protein was determined following the method of Taylor et al. (10). Protein was measured by the methods of Lowry (11).

(e) **Ovulation.** Commencing at the same time as injection of 8 IU PMSG into 28-day-old rats, the control group consisting of nine rats received injections of vehicle, and two experimental groups of five rats each, received either F-1 or F-2 fractions twice a day, at 10.00 and at 17.00, for three days. Both F-1 and F-2 fractions were administered using the same dosage regimen, such that the total dosage per rat was 5.0 mg. At 10.00 on the fourth day, the animals were sacrificed by cervical dislocation, and the number of eggs which had ovulated into the Fallopian tubes was counted under the stereo microscope.

**In vitro experiment**

(a) **Estradiol (E₂) levels.** In these experiments, E₂ levels were measured in the culture medium of ovarian cells which had been exposed to hop extracts in vitro. Ovarian cells were isolated from the ovaries 48 h after PMSG injection, following a procedure as described by Hamberger et al. (12). After isolation, 10⁶ ovarian cells were incubated with 10 ng of FSH (NIADDK-WSH17, 20 UI/mg NIH-FSH-S1) in 1 ml Eagle’s MEM containing vehicle, F-1 or F-2, at 37°C for 20 h in an atmosphere of 95% O₂-5% CO₂. Both F-1 and F-2 fractions were added to the medium using five different dosage regimens, such that the original dosage of the fractions was 500 mg/l and further serial doubling dilutions to 31.3 mg/l were prepared in the medium. The culture medium was centrifuged at 600 × g for 10 min at 4°C, and the supernatant was stored at —30°C until E₂ assays were performed. E₂ levels were assayed by RIA using a kit manufactured by the Daiichi-Radioisotope Co. Ltd. The standard curve ranged from 1.6 to 400 pg/tube. The E₂ values were expressed as pg per 10⁶ cells.

(b) **cAMP levels.** cAMP was measured in the culture medium of ovarian cells which had been exposed to hop.
extracts in vitro. Each aliquot (100 μl) was taken from the culture 3 h after the incubation. The medium was kept frozen at −80°C until assay. Assay was performed with the method of Gilman (13), modified as previously described (14).

(c) LH levels. In these experiments, LH levels were measured in the culture medium of anterior pituitary cells which had been exposed to hop extracts in vitro. Anterior pituitary tissues were removed from 90-day-old rats (average body weight, 340 g) three weeks after ovariectomy, and sliced with scissors. The sliced tissue were incubated in a shaking water bath containing a 0.2% collagenase-0.2% trypsin solution for 40 min at 37°C and were filtered through #60 and #150 nylon meshes. After rinsing in Hank’s solution, the cells thus obtained were suspended in Eagle’s MEM containing vehicle, F-1 or F-2, and incubated for 24 h at 37°C in an atmosphere of 95% O2-5% CO2. The vehicle consisted of 1 ml of MEM. For both F-1 and F-2, two different dosage regimens were used, consisting of either 100 μg or 500 μg dissolved in 1 ml of MEM. The LH levels in the culture medium were assayed by RIA in the same manner as that described for serum LH assays.

**Statistical analysis**

Each point was determined over triplicate in the experiments with cells, blood, uterus and of ovulation. Each column in the figure or table represents mean±SEM. Student’s t-test or the Cochran-Cox test was used for statistical evaluation of the data.

**Results**

**In vivo experiments**

(a) Serum LH levels. As shown in Fig. 1, LH values were expressed as μg/l of serum. Compared to the control value of 284±16 μg/l, serum LH values significantly decreased to 174±8 μg/l (61.3%; p<0.001) in the group treated with 5 mg of hop fraction F-1, and to 144±18 μg/l (50.8%; p<0.001) in the group treated with 5 mg of fraction F-2.

(b) E2 levels. As shown in Fig. 2, E2 values were expressed as ng per pair of ovaries. Compared to the control value of 5.10±0.16 ng, E2 released into the culture medium significantly decreased to 2.81±0.17 ng (54.3%; p<0.001) and 2.31±0.11 ng (44.7%; p<0.001) in the groups treated with, respectively, 0.5 mg and 5.0 mg of hop fraction F-1, and decreased to 2.20±0.14 ng (42.4%; p<0.001) and 2.26±0.21 ng (51.2%; p<0.001) in the groups treated with, respectively, 0.5 mg and 5.0 mg of fraction F-2.

(c) P levels. Fig. 3 shows a comparison of P produced in the culture medium of luteal cells isolated from the control group and from the groups treated with hop fraction F-1 or F-2 and cultured in the presence or absence of hCG. The P values were expressed as ng per 105 cells and then compared with the values in percentage. When cultured in the absence of hCG, in comparison with the control value of 1.44±0.05 ng, P released into the medium significantly decreased to 8.9%.
Fig. 3. Progesterone production in the incubation medium of the luteal cells from the PMSG rats injected with 5.0 mg of F-1 or F-2. Each column represents mean±SEM (N = 6). † p<0.001 compared with saline control without F-1 or F-2 and hCG. * p < 0.001 in F-1, ** p < 0.05 and *** p < 0.01 in F-2 compared with the control without hCG.

Fig. 4. Thymidine kinase activity in the uterine tissue from the PMSG rats injected with 0.5 mg or 5.0 mg of F-1 or F-2. Each column represents mean±SEM (N = 5). * p<0.01 compared with saline control.

Table 1. Effect of hop extracts on ovulation of the PMSG rats. 5.0 mg of F-1 or F-2 at total dosage was given twice daily for 3 days to 28-day-old rats injected with 8 IU of PMSG. Three days later, the rats were killed and the ovulation was checked as described under Materials and Methods. * p<0.05 compared with saline control.

<table>
<thead>
<tr>
<th>Rat no.</th>
<th>Dose (mg) /rat</th>
<th>Body wt (g)</th>
<th>Ova no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>Saline</td>
<td>88.9±2.2</td>
</tr>
<tr>
<td>F-1</td>
<td>5</td>
<td>5</td>
<td>87.0±1.2</td>
</tr>
<tr>
<td>F-2</td>
<td>5</td>
<td>5</td>
<td>85.2±1.5</td>
</tr>
</tbody>
</table>

(p<0.001) in the group treated with hop fraction F-1, and to 11.9% (p<0.001) in the group treated with fraction F-2 as represented by the dagger symbols in the figure. The addition of 10^-5 or 10^-4 IU/1 of hCG significantly increased P levels in the controls. This hCG-induced increase in P was also observed in the groups treated with hop fraction F-1 or F-2, though the baseline was greatly decreased. Compared to their respective controls, which were incubated with either 10^-5 or 10^-4 IU/1 of hCG, P values significantly decreased to 11.3% (p<0.001) and 9.4% (p<0.001) in the groups treated with hop fraction F-1, and to 15.0% (p<0.001) and 12.5% (p<0.001) in the groups treated with fraction F-2. However, it was found that the addition of 10^-4 or 10^-3 IU/1 of hCG significantly increased P levels in these groups when compared with their respective controls, i.e. P release in the absence of hCG in the group treated with F-1 or F-2.

(d) TK activity in the uterine tissue. As shown in Fig. 4, TK activity was expressed as dpm × 10^-5/mg protein. Compared to the control group, TK activity significantly decreased to 78.0% (p<0.01) in the group treated with 5 mg of hop fraction F-1, and to 66.1% (p<0.01) in the group treated with 5 mg of fraction F-2.

(e) Ovulation. As shown in Table 1, compared to the control group value of 12.5±0.2 the number of ovulations observed in the groups treated with hop fractions F-1 and F-2 significantly decreased to values of 8.0±1.2 and 7.2±1.8, respectively.

In vitro experiments

(a) E2 and cAMP levels. As shown in Fig. 5A, FSH-induced E2 values were expressed as pg per 10^6 cells for 20 h incubation at 37°C. Compared to the control value 202.9±18.3 pg, E2 released into the culture medium significantly decreased to 52.1% and 41.3% (both p<0.01 vs control) in the groups treated with, respectively, 125 mg and 250 mg/l of hop fraction F-1, and compared to the control value of 163.6±12.7 pg, E2 value in the culture medium significantly decreased to 48.3% and 53.2% (both p<0.01 vs control) in the
groups treated with, respectively, 125 mg and 250 mg/l of fraction F-2.

As shown in Fig. 5B, FSH-induced cAMP values were expressed as pmol per $10^6$ cells for 3 h incubation at 37°C. Compared to the control value of $4.84 \pm 0.57$ pmol, cAMP into the culture medium significantly decreased to 45.5% and 34.7% (both $p < 0.05$ vs control) in the groups treated with, respectively, 125 mg and 250 mg/l of fraction F-1, and compared to the control value of $6.02 \pm 0.81$ pmol, cAMP value in the culture medium significantly decreased to 39.9% and 23.4% (both $p < 0.05$ vs control) in the groups treated with, respectively, 125 mg and 250 mg/l of fraction F-2. In a control experiment, the addition of F-1 or F-2 fraction in the dose from 31.3 to 500 mg/l into the culture medium, which contains 10 pmol of cAMP, did not influence the cAMP determination (data are not shown).

(b) LH levels. As shown in Fig. 6, LH values were expressed as mg/l/24 h. Compared to the control value of $5.7 \pm 0.2$, neither F-1 nor F-2 caused any significant difference in LH release into the culture medium of anterior pituitary cells.
Discussion

We have previously reported that PMSG-induced increases in the ovarian weights of immature rats were found to be suppressed by an activity in the residues after removal of the resins and chlorophyll with acetone from the hop cone (5). We identified two fractions with high activity and designated them F-1 and F-2. The present experiments were aimed at studying the effects of these hop extracts on the stimulatory actions of exogenous PMSG in relation to LH release, production of E2 and P, and uterine TK activity in immature rats. We also examined whether these extracts act directly on the pituitary or on the ovary.

Research to detect the presence of estrogenic compounds in hop extracts has been conducted since changes in the menstrual cycle or mammary glands of women engaged in the cultivation of hops were frequently observed. Koch et al. (1) reported that 100 g of hops contain 200–300 mg of estrogen-like compounds, as measured by the method of Allen-Doisy. Furthermore, Zenisek et al. (2) and Churý (3) noted estrogenic activity in hop extracts when saponified hops were extracted with 95% ethanol. However, Fenselau et al. (4) reported that no estrogenic activity was observed upon measurement of uterine weights of mice administered with 0.003–30 mg of saponified hops extracted with ethanol, hop extracts prepared with organic solvents such as methanol, methylene chloride, and hexane, or purified hop oils and resin constituents prepared from Yakima, Hallett, or Zotec strains of hops indigenous to Western countries.

Previously, we have reported that, in the course of investigating the biological activity of water-soluble compounds remaining in the residue after eliminating lipid-soluble constituents of hops, the increase in ovarian weight of immature rats induced by PMSG injections was found to be inhibited by these hop extracts. In the present study, we have further studied the effects of hop extracts on other parameters in the pituitary-gonadal system. We have previously reported that ovarian weight increases after injection of PMSG into immature female rats, and that two days after injection an LH surge is detected, on the third day after injection ovulation is observed, and on the sixth day after injection secretion of P from the corpus luteum reaches maximum (6).

In the present experiments, serum LH levels were examined on the fourth day after PMSG injection, and a significant decrease in serum LH was noted in the groups treated with 5 mg of hop fraction F-1 or F-2 when compared to controls. In order to determine whether or not this decrease in LH levels was the result of a direct action of the hop extracts on the pituitary, the extracts were added to the medium of anterior pituitary cells in culture. Cells were isolated from the pituitary glands of rats whose gonadotropin secretion had been stimulated to high levels by bilateral ovariectomy. However, the LH levels in the culture medium after treatment with hop extracts in vitro showed no significant difference compared to those of controls consisting of pituitary cells cultured alone without any additives. Thus, it is difficult to imagine that the decrease in serum LH levels observed upon injection of hop extracts in vivo was the result of inhibition of LH secretion due to a direct action on the anterior lobe of the pituitary gland.

The E2 levels in medium from ovarian cells cultured from rats, which had been injected with hop extracts for two days commencing at the same time as the PMSG injection, were found to be significantly decreased compared to controls. Furthermore, E2 and cAMP levels in the medium of ovarian cells from rats on the second day after injection of PMSG alone, i.e. just prior to ovulation, significantly decreased by the addition of 1.25 μg of either F-1 or F-2 in vitro. This indicates that hop extracts antagonize the stimulatory effect of PMSG on E2 secretion from granulosa cells at the ovarian site via the adenylate cyclase system. Because the antagonistic effect on estrogen secretion would prevent a rise in serum estrogen levels, it is probable that serum LH levels were also lowered as a result. In addition, when the uterus was removed 30 h after simultaneous injection of PMSG and 5 mg of either F-1 or F-2, uterine TK activity was found to be significantly suppressed compared to controls. The serum E2 levels of immature rats injected with PMSG start to rise rapidly 20 h after injection (15). We have previously reported that when E2 is injected into immature rats, the TK activity in the uterus of these rats rises markedly 30 h later (16). In the present study, this rise in TK activity was inhibited in the animals which had been injected with hop extracts; the implication is that this phenomenon was indeed the result of inhibition of E2 secretion by the hop extracts. In addition, when culturing luteal cells from rats pretreated with PMSG, P secretion is markedly increased if hCG is added to the culture medium. In the present study, when hop extracts were injected along with PMSG, the basal secretion of P from the rat luteal cells into the culture medium decreased to less than 1.2% of the controls, yet P secretion did rise significantly in response to hCG addition, as shown in Fig. 3. Thus the hop extracts antagonized the action of PMSG, but did not inhibit the response to hCG.

Furthermore, in the experimental groups injected with the hop extracts, the number of ovulations seen on the third day after PMSG injection was significantly decreased. Again, this is thought to be due to the hop extracts inhibiting PMSG-induced E2 secretion from the ovary, resulting in a decrease in the LH surge.

Administration of the fractions to PMSG rats did not suppress the increase in the weights of body, liver and spleen in vivo. Neither did the fractions inhibit LH secretion from pituitary in vitro. These results indicate that the hop extracts are not cytotoxic to the rat body and its endocrine organs. Therefore, we think that inhibition of the FSH-induced granulosa cell proliferation is a specific activity of the fractions. And this might
lead to lowered E₂ production. LH secretion, ovulation and ovarian weight. Exposure of ovarian cells either in vivo or in vitro to the fractions did not lower their viability in culture resulting in decreased responses.

In conclusion, in the present experiments employing immature female rats, hop extracts inhibited the rise in E₂ due to PMSG injection, and also inhibited the increase in serum LH levels. These lowered E₂ and LH concentrations caused the inhibitory effects on ovarian weight, uterine TK activity, ovulation, and P secretion in the PMSG treated rats. The present experiments indicate that the LH inhibition by hop extracts is not mediated via the pituitary gland. We have also obtained results suggesting that hop extracts do not inhibit the responsiveness of luteal cells to hCG (LH), but that the extracts do inhibit the response of the ovaries to PMSG (FSH).

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