Regulative effects of ovarian steroids on rat gastric motility and sensitivity

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Abstract: Women often complain gut symptoms during pregnancy and the luteal phase of the menstrual cycle. To investigate the relationship between ovarian steroids and the abnormal gut motility and sensitivity, the expressions of cholecystokinin (CCK), calcitonin gene-related peptide (CGRP) and their receptors in stomach were studied in ovariectomized rats. Blood samples were collected for estradiol (E2), progesterone (P4), CCK and CGRP radioimmunoassay. Expression of CCKA receptor in fundus was assessed by Western blot and CGRP receptor was determined by 125I-CGRP radioligand binding assay (RBA). The replacement therapy with estradiol benzoate (EB) could dose-dependently increase the plasma CCK level and the expression of gastric CCKA receptor (P<0.05 respectively). P4 replacement therapy could stimulate the release of CGRP and increase the binding sites of CGRP receptors in stomach (P<0.05 respectively). The combined effect of EB and P4 was to stimulate the release of CCK and CGRP, and to increase the expressions of gastric CCKA and CGRP receptors. These results indicate that EB could inhibit gastric emptying by increasing CCK secretion and CCKA receptor expression in ovariectomized rats. P4 could increase gut sensitivity by up-regulating the release of CGRP and the activity of CGRP receptor. It could be deduced from these observations that CCKA and CGRP receptor antagonists could be used for female patients who suffer from gastrointestinal dysfunction closely related with the menstrual cycle, such as distension, satiety, bloating and abdominal pain.

Key words: estradiol; progesterone; receptor; cholecystokinin; calcitonin gene-related peptide

Women often complain more symptoms of disordered gut function such as nausea, vomiting, abdominal pain, distension, satiety, bloating, diarrhea or constipation, especially during pregnancy or the luteal phase of the men-
strual cycle\[1\]. Furthermore, 2/3 patients of irritable bowel syndrome (IBS, one kind of gut dysfunctional disease) are female and among these patients, vomiting, constipation and bloating are the most familiar symptoms. But male patients suffer from more symptoms of diarrhea\[2\]. These clinical observations have led to the speculation concerning the possible effects of ovarian sex steroids on gastrointestinal function. Several studies have shown that estradiol (E\(_2\)) decreases gastric motility and intestinal transit\[1,3\]. But the mechanism involved in these effects has not been elucidated. Cholecystokinin (CCK) is an important brain-gut peptide involved in the modulation of pancreatic enzyme release and the inhibition of feeding and gastric emptying. There are two kinds of receptors responsible for its function — CCK\(_A\) and CCK\(_B\). The previous studies have suggested that CCK\(_A\) receptors play an important role in the inhibition of gastric emptying by amphetamine or endogenous and exogenous CCK\[4\]. Calcitonin gene-related peptide (CGRP) is an important sensory neurotransmitter in sensing different stimulation in the gut. CGRP receptors have been found to express in the dorsal root ganglia neurons and the myenteric plexus\[5\]. The present study examined the effects of estradiol benzoate (EB), progesterone (P\(_4\)), the coordinated EB and P\(_4\), replacement therapy on plasma CCK and CGRP secretion in ovariectomized rats. At the same time, the expressions of CCK\(_A\) and CGRP receptors in rat stomach tissue were also determined to further investigate the mechanism involved in the modulation of gut motility and sensitivity by the ovarian steroids.

1 MATERIALS AND METHODS

1.1 Animals

Female Sprague-Dawley rats weighing 250–300 g were housed at room temperature and fed with rat chow. Tap water was given \textit{ad libitum}. Animals were divided randomly and every 6 rats were housed in one cage. Animal protocols were approved by the Animal Care Institution of Shandong University.

1.2 EB and P\(_4\) replacement protocol

1.2.1 EB replacement

Twenty-four female rats were randomly divided into 4 groups and were ovariectomized bilaterally under light ether anesthesia 2 weeks before the subcutaneous injection of EB (4, 10, or 25 µg/kg)\[6\] in the dorsum of the rat once daily for 6 d. The control animals were injected with the same volume of sesame oil. Twenty-four hours after the previous injection, the rats were fasted overnight. In the next morning the rats were decapitated, the uteri were weighed and blood samples were collected for E\(_2\), P\(_4\), CCK and CGRP radioimmunoassay (RIA). The fundus tissues were collected for the expressions of CCK\(_A\) receptor by Western blot and CGRP receptor by \(^{125}\)I-CGRP radioligand binding assay (RBA). On the day of the experiments, another 6 intact rats at the diestrus stage, verified by vaginal smears, were employed as normal control group (NC).

1.2.2 P\(_4\) replacement

Twenty-four female rats were randomly divided into 4 groups and were ovariectomized bilaterally under light ether anesthesia 2 weeks before the subcutaneous injection of P\(_4\) (10, 20, or 40 mg/kg)\[6\] in the dorsum of the rat once daily for 6 d. The control animals were injected with the same volume of sesame oil. Twenty-four hours after the previous injection, the rats were fasted overnight. In the next morning the rats were decapitated, the uteri were weighed and blood samples were collected for E\(_2\), P\(_4\), CCK and CGRP RIA. The fundus tissues were collected for the expressions of CCK\(_A\) receptor by Western blot and CGRP receptor by \(^{125}\)I-CGRP RBA.

1.2.3 \(EB+P_4\) replacement

Twenty-four female rats were randomly divided into 4 groups and were ovariectomized bilaterally under light ether anesthesia 2 weeks before the subcutaneous injection of EB (10 µg/kg), P\(_4\) (20 mg/kg), or EB (10 µg/kg)+P\(_4\) (20 mg/kg) in the dorsum of the rat once daily for 6 d. The control animals were injected with the same volume of sesame oil. Twenty-four hours after the previous injection, the rats were fasted overnight. In the next morning the rats were decapitated, the uteri were weighed and blood samples were collected for E\(_2\), P\(_4\), CCK and CGRP RIA. The fundus tissues were collected for the expressions of CCK\(_A\) receptor by Western blot and CGRP receptor by \(^{125}\)I-CGRP RBA.

1.3 Measurement of uterine weights

Since uterus is stimulated by the ovarian sex steroids, uterine weight in each treatment group was measured to confirm the biological effects of EB and P\(_4\). After decapitation, rat uterus was removed, defatted, cleaned, drained of fluid, blotted on filter paper and weighed.

1.4 \(E_2\), \(P_4\), CCK, and CGRP RIA

After decapitation, rat blood samples were collected into a cold tube (with 10% EDTA and 500 kIU/ml aprotinin in it). Plasma was immediately prepared by centrifugation at
2 000 g for 20 min at 4 °C and stored at −70 °C for measurement of plasma CCK and CGRP concentrations. For measurement of plasma E2 and P4 concentrations, rat trunk blood was collected after decapitation. Plasma was separated by centrifugation at 1 000 g for 25 min and stored at −20 °C. RIA operation was done according to the experimental protocol being provided.

1.5 Protein extraction and Western blot analysis for CCKα-receptor

Proteins were extracted using TRIzol reagent from the collected biopsy samples. Approximately 20 µg of cellular protein extracts were separated electrophoretically on 10% SDS polyacrylamide gel and transferred onto Aque-Blot™ PVSF membrane (BioRad, USA) by electroblotting. Following protein transfer, the protein membrane was incubated in blocking buffer (5% defatted milk, 137 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.6, 0.1% Tween-20) for 1 h at room temperature. The membrane was then incubated with goat polyclonal anti-CCKα receptor (1:100, Santa Cruz). β-actin was used for positive control and comparative quantification. The incubation was followed by 3 washes in blocking solution for 10 min each. The appropriate secondary anti-goat IgG conjugated with horseradish dioxxygenase (1:6 000; Santa Cruz) diluted in blocking solution were incubated with protein membrane for 60 min, followed by 3 washes 10 min each. The membrane was then incubated in the visualization solution (ECL reagent), followed by the exposition to an X-ray film (Kodak, Wiesbaden, Germany). The film was scanned by PDS1 scanner and analyzed by Personal Densitometer S1 software. The quantification of CCKα receptor was expressed as the densitometry ratio of CCKα receptor to β-actin.

1.6 125I-CGRP RBA

The fundus tissues were homogenized in 50 mmol/L Tris-(hydroxymethyl) aminomethane buffer (pH 7.4, containing 0.32 mol/L sucrose, 1 mmol/L dithiothreitol, 5 mmol/L EDTA, and 200 kIU/L of aprotinin), and the homogenate was centrifuged at 800 g for 10 min. The protein in the membrane pellet was quantified by Lorry method. Membrane preparations were incubated in 600 µl of total reaction volume at 37 °C for 4 h with 6.4×10−12 mol/L 125I-CGRP (labeled by Iodogen method[7], specific activity: 2 400 Ci/mmol). Specific binding was calculated from the total amount of labeled CGRP binding minus the non-specific binding amount in the presence of 0.5 mmol/L unlabeled CGRP. The data were analyzed with the Scatchard method and the results expressed as CGRP binding (in fmol/mg) to the membrane protein.

1.7 Drugs

Chemicals used in the study included: estradiol benzoate, progesterone, sesame oil, EDTA, aprotinin, dithiothreitol and sucrose were purchased from the Sigma Chemical Company. RIA kits for E2, P4, CCK, and CGRP were purchased from Northern Biological Corporation of Beijing, China. 125I and CGRP were purchased from East Asia Immunotechnique Company, China.

1.8 Statistical analysis

Results were expressed as means±SEM, with n referring to the number of animals. Data were analyzed for statistical differences with the Student two-tailed t-test. P<0.05 was considered to be significant.

2 RESULTS

2.1 Effects of EB, P4 on uterus weight, plasma CCK and CGRP in ovariectomized rats

The uterus weight of ovariectomized rats was decreased without EB and P4 replacement. After the administration of EB and P4 for 6 d, the uterus weight increased dose-dependently. Especially in the EB treatment group, the uterus weight restored nearly to the normal control level. The plasma CCK concentration was increased by higher doses of EB (10, 25 µg/kg) administration while EB had no effect on the plasma CGRP. P4 could increase the plasma CCK concentration to some extent but the difference was not statistically significant. The plasma CGRP secretion was higher with P4 administration than that in the vehicle group. Another interesting finding was that EB replacement increased the plasma P4 concentration while P4 administration did not have any effect on the plasma EB (Table 1).

2.2 Effects of EB, P4 on gastric CCKα receptor expression in ovariectomized rats

The Western blot results indicated that EB dose-dependently up-regulated the expression of CCKα receptor while P4 did not have any effect on it in ovariectomized rats’ fundus. The coordinated effect of EB and P4 administration was to increase the CCKα receptor expression (Fig.1 and Fig.2).

2.3 Effects of EB, P4 on gastric CGRP receptor binding sites in ovariectomized rats

There are two types of CGRP receptors and both of them are supposed to be responsible for CGRP as a vasodilator and neurotransmitter. So we performed 125I-CGRP binding studies to assess the effect of ovarian steroids on the CGRP binding sites in ovariectomized rats’ stomach tissue. EB did not affect the CGRP binding sites while P4 dose-dependently increased the activity of CGRP receptor (P<
Table 1. Uterus weight, plasma E2, P4, CCK and CGRP in ovariectomized rats after the administration of EB and P4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uterus weight (mg)</th>
<th>E2 (pg/ml)</th>
<th>P4 (ng/ml)</th>
<th>CCK (pg/ml)</th>
<th>CGRP (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>402±28</td>
<td>24.0±2.6</td>
<td>30.6±1.7</td>
<td>45.5±1.4</td>
<td>208.6±23.1</td>
</tr>
<tr>
<td>Ovx+oil</td>
<td>109±8</td>
<td>5.2±0.9</td>
<td>4.8±0.6</td>
<td>35.6±3.5</td>
<td>109.9±12.3</td>
</tr>
<tr>
<td>Ovx+EB (4 µg/kg)</td>
<td>326±18</td>
<td>24.8±3.6</td>
<td>10.2±0.8*</td>
<td>42.6±4.5</td>
<td>120.2±12.8</td>
</tr>
<tr>
<td>Ovx+EB (10 µg/kg)</td>
<td>389±16*</td>
<td>52.6±4.8</td>
<td>15.8±1.2*</td>
<td>56.6±4.8*</td>
<td>106.4±9.5</td>
</tr>
<tr>
<td>Ovx+EB (25 µg/kg)</td>
<td>406±19*</td>
<td>82.6±12.4</td>
<td>18.4±1.6*</td>
<td>68.8±10.4*</td>
<td>114.4±13.3</td>
</tr>
<tr>
<td>Ovx+oil</td>
<td>132±6</td>
<td>5.8±0.9</td>
<td>5.6±0.5</td>
<td>32.2±2.6</td>
<td>96.8±8.4</td>
</tr>
<tr>
<td>Ovx+P4 (10 mg/kg)</td>
<td>186±12</td>
<td>6.2±1.2</td>
<td>20.3±1.6</td>
<td>36.5±3.2</td>
<td>148.5±12.2*</td>
</tr>
<tr>
<td>Ovx+P4 (20 mg/kg)</td>
<td>178±8*</td>
<td>6.8±1.4</td>
<td>32.6±1.8</td>
<td>31.8±2.5</td>
<td>169.9±14.7*</td>
</tr>
<tr>
<td>Ovx+P4 (40 mg/kg)</td>
<td>189±11</td>
<td>6.3±1.6</td>
<td>48.5±3.5</td>
<td>36.6±3.8</td>
<td>198.7±21.0*</td>
</tr>
<tr>
<td>Ovx+oil</td>
<td>122±6</td>
<td>5.6±0.6</td>
<td>4.8±0.6</td>
<td>30.5±2.2</td>
<td>98.8±10.4</td>
</tr>
<tr>
<td>Ovx+EB (10 µg/kg)</td>
<td>486±31*</td>
<td>50.4±4.2</td>
<td>9.8±0.8</td>
<td>53.2±3.6*</td>
<td>94.5±8.2</td>
</tr>
<tr>
<td>Ovx+P4 (20 mg/kg)</td>
<td>146±8</td>
<td>6.4±1.1</td>
<td>36.4±2.2</td>
<td>28.8±2.1</td>
<td>189.6±12.3*</td>
</tr>
<tr>
<td>Ovx+P4 (40 mg/kg)</td>
<td>328±22*</td>
<td>48.8±4.0</td>
<td>38.5±3.2</td>
<td>48.4±3.9*</td>
<td>167.7±15.4*</td>
</tr>
</tbody>
</table>

E2, estradiol; P4, progesterone; EB, estradiol benzoate; NC, normal control group in diestrus stage; Ovx, ovariectomized rat. *P<0.01 vs Ovx+oil, #P<0.05 vs Ovx+oil. n=6.

Fig.1. Representative Western blot showing CCKA receptor expression from fundus preparation after different drug treatments.

Fig.2. Dose-response effects of estradiol benzoate (EB), progesterone (P4) and the coordinated effect of EB and P4 on CCKA receptor expression in the fundus of ovariectomized rats. A: EB dose-dependently increased CCKA receptor expression in the fundus of ovariectomized rats. *P<0.05 vs vehicle-injected rats. B: P4 had no effect on CCKA receptor expression in the fundus of ovariectomized rats. C: Coordinated effect of EB and P4 was to increase CCKA receptor expression in the fundus of ovariectomized rats. *P<0.05 vs vehicle-injected rats.
0.05 vs vehicle group). The coordinated administration of EB and P₄ increased the CGRP binding sites to the same extent as P₄ did (P<0.01 vs vehicle group, Fig.3).

3 DISCUSSION

Gender difference is one important character of many gut functional diseases such as IBS[9]. Studies about the mechanism of gender difference in the pathogenesis of these diseases indicate that man and woman have different physiological reaction, which could influence the gastrointestinal transit time, gut sensitivity and pain treatment in the central nervous system[2,8,9]. Various observations suggest that the fluctuations in sex hormones in women may have an influential role in gut motility and sensitivity which would lead to the symptom exacerbation such as diarrhea, bloating or abdominal pain during some phase of the menstrual cycle or pregnancy. Animal studies have shown increased responsiveness to visceral stimuli as measured by visceral motor responses or greater autonomic manifestations during the metestrus/diestrus cycle phase[10,11], which corresponds to the perimenstrual phase in women.

Estrogen and progesterone have direct effects on visceral organs as well as the peripheral and central nervous systems. Estrogen and progesterone can act alone or in combination influence both gut function and symptom experiences. Different mechanisms have been proposed for these effects of EB in the gastrointestinal tract. By using CCKₐ receptor antagonist, the effect of EB-induced inhibition of gut motility was abolished[4]. Our present study directly examined the expression of CCKₐ receptor in ovariectomized rats and the results also supported that EB could inhibit the stomach motility by increasing the CCK secretion and CCKₐ receptor expression. These results indicated there was some cross-action between steroid hormones and enteric neural endocinical system. Apart from the relationship with CCK, estrogen has direct effects on calcium-dependent potassium channels, thus affecting contractility. Furthermore, estrogen could regulate adenosine triphosphate-induced increase in intracellular calcium, resulting in inhibition of primary afferent firing. Estrogen may also centrally modulate perceptual and autonomic responses via an oxytocin-mediated increase of opioid and α₂-adrenoreceptor activity, respectively[12]. Taken together, these results suggested that estrogen may decrease visceral sensory perception and gut motility.
Our previous data indicated that P4 could stimulate rather than inhibit gastric emptying (data not shown). Since P4 didn’t have any effect on the plasma CCK and gastric CCKA receptor expression, this stimulating effect of P4 on gastric emptying may be not through CCK pathway. And the effect of P4 on gastric CGRP receptor expression indicated that P4 could influence the gut motility and sensitivity through CGRP mechanism because CGRP is an important neurotransmitter of the enteric nervous system which could influence the gut motility, secretion and sensation. Other studies about the effect of P4 also suggested that the mesenteric artery CGRP receptors were elevated during pregnancy and decreased at term\[12,13\]. Furthermore, treatment with antiprogestosterone RU-486 causes decrease of CGRP receptors, whereas P4 treatment during late gestation maintains CGRP receptors in resistance blood vessels at the similar levels to those observed during pregnancy\[14\]. Since CGRP is not only a potent vasodilator, but also an important sensory neurotransmitter, the up-regulation of CGRP receptor would not only increase the blood supply to the stomach but also strengthen the afferent signal transduction to the central nervous system. The coordinated effects of these actions would result in the hypersensitivity to the stimulation in the gut.

The modulation of ovarian steroids on the expressions of CCKA and CGRP receptors in the stomach provides a new mechanism for the therapy of gut dysfunction. The antagonists for CCKA and CGRP receptors may stimulate the gut motility and decrease the abnormal hypersensitivity in IBS patients who suffer from bloating, satiety, abdominal pain and constipation. On the other side, the stimulator for CCKA receptor may inhibit the gut motility so that it could be used for patients suffering from diarrhea. In a word, the exploration of specific antagonists or stimulators for CCKA and CGRP receptors could be beneficial for female patients who suffer from gut dismotility or hypersensitivity, especially those exacerbating in some special phase of the menstrual cycle.

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REFERENCES


