Ovarian hormone modulates 5-hydroxytryptamine 3 receptors mRNA expression in rat colon with restraint stress-induced bowel dysfunction

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AIM: To examine the effects of ovarian hormone on the expression of 5-hydroxytryptamine 3 receptors (5-HT, R) in rat colon of restraint stress-induced bowel dysfunction.

METHODS: Twenty-four female Sprague-Dawley rats were randomly divided into three groups of 8 each: sham operation, ovariectomy (OVX) and OVX+E2+P group, OVX group showed increase in fecal pellets and decrease in the time of voidus pellets excretion (P<0.01). Serum levels of E2 and P were suppressed in OVX group and restored following treatment with ovarian steroids (P<0.01), and the levels of 5-HT, R mRNA in the colon of ovariectomized rats were significantly increased. The expression of 5-HT, R mRNA was significantly decreased in hormone replacement therapy group (P<0.01).

CONCLUSION: Ovarian hormone plays a role in the regulation of 5-HT, R expression in restraint stress-induced bowel dysfunction of rats. The interactions between ovarian steroids and gastrointestinal tract may have major pathophysiological implications in 5-HT-related disorders, such as irritable bowel syndrome (IBS).

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INTRODUCTION

IBS is characterized by intermittent or continuous abdominal pain and alterations in bowel patterns[1-2]. The syndrome is one of the most common gastrointestinal (GI) disorders seen in primary care and specialist practices[3-4]. There is a female-to-male ratio of 2/1 in North American population studies of IBS[5], and epidemiologic surveys indicate that women seek health care services for IBS more frequently than men[6,7]. In menstruating women, symptoms are influenced by menstrual cycle. Peri- and postmenopausal women have a high prevalence of altered bowel function and IBS-like gastrointestinal complaints[8]. For many women, symptoms become amplified around the time of menses[9]. However, the exact mechanism (e.g. ovarian hormones, stress response) accounting for these cyclic changes is still unknown.

Studies suggested that the female sex steroid hormones, including estrogen and progesterone, could affect the myoelectric and mechanical activity of colonic smooth muscle in vitro. By geometric center method, estrogen and progesterone pretreatment of ovariectomized rats resulted in a significant decrease in colonic transit compared with untreated ovariectomized rats[10]. 5-HT, R is a ligand-gated ion channel and probably involved in the modulation of colonic motility and visceral pain in the gut[10]. Clinically, 5-HT, R antagonists are important in the treatment of symptoms in IBS and more effective on diminishing bowel pattern symptom in women as compared to men[11]. Rat with wrap restraint stress is an appropriate animal model to study stress-related colonic dysfunction like IBS[12]. Previous studies have suggested that restraint stress results in an increase of fecal pellet output in rats via peripheral 5-HT, receptors[13].

Earlier research has shown that E2 can suppress gastric motility response to thyrotropin-releasing hormone (TRH) and restraint stress in conscious rats[14], low dose of P (1 mg/kg) increased intestinal transit while higher dose (10-20 mg/kg) had no effect. Recently, it has been demonstrated that both forskolin and 17-beta-estradiol inhibit the function of 5-HT, R in a noncompetitive manner and that this inhibition is independent of cAMP levels[15]. Based on the research mentioned above, we suggested that this change, in part, be due to the increased activity of 5-HT, R innervating these organs. Therefore, we decided to assess whether both of these sex steroids could modulate mRNA expression of 5-HT, R in rat colon with restraint stress-induced colonic dysfunction and the potential mechanisms involved. Ovariectomized rats were treated with or without E2 and P in combination, after that changes in fecal pellets and the time of voidus pellets output were assessed. Furthermore, we used RT-PCR to determine whether there was a decrease in 5-HT, R mRNA expression after treatment with sex steroids.

MATERIALS AND METHODS

Animals

Twenty-four adult female Sprague-Dawley rats (weighing 220-250 g), purchased from the Animal Department of Tongji Medical College, Huazhong University of Science and Technology were housed individually in a light and temperature controlled room with light-dark cycles of 12:12 h, where the temperature (24±2 °C) and relative humidity (65-70%) were kept constant. The animals were fed on a standard pellet diet, and food was withdrawn overnight before surgery and emptying experiments, but free access to water was allowed ad libitum. Experimental protocols followed standards and policies of the Animal Care and Use Committee, School of Medicine, Wuhan University.
Surgical procedures
Rats were housed for 7 d before experiments. At 6 wk of age, rats were randomly divided into three groups of eight each: sham-operated group, OVX group and OVX+E2+P group. Under aseptic conditions, bilateral ovariectomy or sham operation was performed under general anesthesia with ketamine (100 mg/kg, ip). For OVX, two dorsolateral incisions, in the skin and the peritoneum, were made and the ovaries and uterine horn removed. Sham operations consisted of skin and peritoneum incisions. After operation, animals were housed four per cage under previous conditions. On the same day, rats in OVX+E2+P group were subcutaneously injected a mixture of estradiol benzoate (E2, 5 µg/d) and progesterone (P, 0.2 mg/d). Four weeks after E2 and P combination treatment, wrap restraint stress experiments were performed in the three groups.

Restraint stress-induced bowel dysfunction
The method described by Williams et al. [16] was used with slight modifications. Rats were acclimated to metal mesh cage placed on a tray for 5 h (9:00-14:00). After the acclimation, they were lightly anesthetized with ether. Then, their forelimbs were restrained with adhesive tapes (12.5 mm in width). The restrained limbs and thoracic trunk were wrapped with other adhesive tapes (50 mm in width). Immediately after these rats were returned to the cages, the number of feces dropped on the tray was counted 1 h after the wrapping. Test compounds were administered 1 h or 30 min before the restraint.

Homemone assays
The blood was collected from the heart of anesthetized animals and serum was separated by centrifugation at 1,500 r/min for 20 min and kept at -70 °C until assayed for E2 and P. The gonadal steroids were measured in duplicate by radioimmunoassay using kits (Tianjin Depu Biotech). Aliquots of serum were added to tubes that had been coated with antibodies to steroid hormone followed by addition of 125I-hormone. Mixtures were vortexed gently and incubated for 2-3 h at room temperature. Incubation was terminated by aspirating excess of 125I-hormone and tubes were counted.

RNA extraction and RT-PCR
Distal colon tissues were harvested from animals in various treatment groups and frozen in liquid nitrogen. Total RNA from the frozen tissues was extracted using TRIzol as described by the manufacturer’s protocol (Invitrogen). RNA was dissolved in RNase-free water and the concentration was determined by measuring the optical density (A260 nm). The purity of the RNA was assessed by the ratio of A260/A280. Two micrograms of total RNA from each sample was transcribed into cDNA using M-MLV reverse transcriptase (Promega). Briefly, total RNA was mixed with M-MLV reverse transcriptase (200 U/µL), oligo (dT)15 (50 μmol/L, Sangon, Shanghai), RNase inhibitor (40 U/µL), and dNTPs (1.25 mmol/L each, TaKaRa, Dalian) in a buffer containing 37.5 mmol/L KCl, 25 mmol/L Tris-HCl, and 1.5 mmol/L MgCl2 (pH 8.3) in a total volume of 25 µL. The mixture was incubated at 42 °C for 90 min, heated to 95 °C for 5 min and cooled to 58 °C for 5 min. The resulted cDNA samples were amplified by PCR using Biometra PCR machine (Standard Power Pack P25, Germany) and the following specific primer pairs were used (SBS Genetech, Beijing): rats 5-HT-R sense, 5'-GAG ACC TTC ATT TTC GTG CAG CTT GTG CA-3'; antisense, 5'-ACA GCA GCG TGT CCA GCA CAT ATC CCA CCA-3', for the 397-bp product[17]. Amplification of the rat β-actin gene transcript was used to control the efficiency of RT-PCR among the samples, β-actin sense, 5'-GTC ACC CAC ACT GTG CCC TTC T-3'; antisense, 5'-ACA GAG TAC TTG CCA GGA G-3' for the 542-bp product[18]. PCR mixes contained 25 pmol/L each of sense and antisense primers, 2.0 U Taq DNA polymerase (Biostar), the buffer supplemented with 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs and 1 µL cDNA in 25 µL. The amplification cycles were carried out under the following conditions: first denaturing at 94 °C for 3 min, then denaturing at 92 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s for a total of 35 cycles, finally a 7 min extension at 72 °C was conducted. The resulted PCR products were analyzed on 2% (w/v) agarose gels in TBE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA; pH 8.3) containing 0.05 µg/mL ethidium bromide. Appropriate molecular weight markers (100 bp ladder, MBI) were used to verify the required size of the final PCR product. The gels were scanned and the mean density of the products were visualized and photographed under the BIO-PROFIL (VILBER LOURMAT, France).

Statistical analysis
Results are expressed as mean±SD. The statistical significance of difference was evaluated with one-way analysis of variance (ANOVA), and analyzed by SPSS 10.0. A confidence level of P<0.05 was considered significant.

RESULTS
Fecal pellets output
Table 1 shows the effects of E2+P combination and blank control on restraint stress-induced fecal pellet output in rats. The number of fecal pellets counted during observation period was negligible in unrestrained normal rats. In the restraint stress rats, fecal pellet output was measured with pellet counts of 5.6±0.5 for ovarian hormone-induced sham group. The number of fecal pellets was significantly increased in OVX vehicle-treated group compared with sham and E2+P-treated group (vehicle-treated, 8.6±0.6; sham group, 5.6±0.5; E2+P-treated, 4.1±0.5; P<0.01). Meanwhile, the number of vitreous pellets output was significantly decreased (P<0.01). The number of fecal pellets was significantly decreased (E2+P-treated, 4.1±0.5; sham group, 5.6±0.5; P<0.01) and the number of vitreous pellets output was significantly increased in E2+P-treated group (P<0.01).

Table 1  Effects of ovarian hormone on fecal pellets output in the ovariecctomized restraint stress rats (mean±SD, n = 8)

| Group            | Fecal pellets output (number) | Time of vitreous pellet output (min) |
|------------------|--------------------------------|-------------------------------------|
| Sham-operated    | 5.6±0.5                        | 4.8±0.26                            |
| OVX              | 8.6±0.5                         | 1.3±0.2                            |
| OVX+E2+P         | 4.1±0.5                         | 5.8±0.29                            |

*P<0.000 vs sham-operated group; ^P=0.000 vs sham-operated group.*

| Group            | E2 (pmol/L) | P (nmol/L) |
|------------------|-------------|------------|
| Sham-operated    | 104±4.6     | 26±1       |
| OVX              | 15.7±0.8    | 3.2±0.6    |
| OVX+E2+P         | 168±8.8     | 44±2.8     |

*P=0.000 vs sham-operated group; ^P=0.000 vs sham-operated group.*

Serum concentration of estrogen and progesterone
As expected, serum levels of E2 were suppressed in OVX rats and restored after treatment with ovarian steroids (sham-operated, 104±6; OVX: vehicle-treated 15.7±0.8; E2+P-treated 168±8; P<0.01, Table 2). Serum levels of P were also decreased...
Results in pre- and postmenopausal women taking sex HRT replacement had rates of solid emptying similar to those of men [24].

A decreased rate of gastric emptying of solids compared with being treated with sex hormone-replacement therapy (HRT) had a role in modulating these effects as postmenopausal women treated with E2+P had a trend of lower expression of 5-HT3R mRNA in colon compared with other menstrual-cycles in women [22]. E2 suppresses gastric motility response to thyrotropin-releasing hormone (TRH) and restraint stress in women with IBS [32]. Furthermore, 5-HT3R mRNA expression decreased in OVX rats and restored following chronic treatment with ovarian steroids. Meanwhile, OVX vehicle-treatment significantly increased the number of fecal pellets, more than the time of mucous pellets output. Our study indicated that E2 and P could relieve the rat colon contractile response to restraint stress, thus decrease the number of fecal pellets and increase the number of fecal pellets output.

**Expression of 5-HT3R in the colon of restraint stress rats**

Effects of ovarian hormone on the expressions of 5-HT3R mRNA in colon tissues of restraint stress-induced rats were examined by RT-PCR as shown in Figure 1, which revealed a marked increase in OVX vehicle-treated group (relative optical density: 1.12±0.07). The expression of 5-HT3R mRNA in colon was increased to a maximum in OVX vehicle-treated group (sham: 0.85±0.06, P<0.01) and significantly decreased after E2+P-treatment (0.65±0.05, P<0.01, Figure 1B). Furthermore, 5-HT3R mRNA expression was decreased by 1.3-fold in E2+P-treated group (P<0.01 compared with sham group, Figure 1B). However, there was a trend of lower expression of 5-HT3R mRNA in colon with the increasing serum levels of E2 and P.

**DISCUSSION**

IBS is a complex GI disorder with a poorly understood pathophysiology in which three major mechanisms interact: altered gastrointestinal motility, increased sensory function of the intestine and psychosocial factors [89]. Various observations suggested that fluctuations in sex hormones in women might have an influential role in IBS: for many women, symptoms become amplified around the time of menses [89]. In menstruating women, symptoms are influenced by menstrual cycle and bloating, abdominal pain, and diarrhea tend to be amplified during the late luteal and early menses phases [26,30]. GI symptoms increase and intestinal transit decreases during pregnancy (a time of high E2 and P levels) [26,30], and rectal sensitivity is greater during menses compared with other menstrual-cycles in women with IBS [90]. The precise mechanism responsible for the changes in gastrointestinal motility is still unknown. Earlier research has shown that E2 can delay gastric emptying and GI transit in rats [26,30], low dose of P (1 mg/kg, i.p.) enhanced the gastric emptying and high dose of P (5 mg/kg, i.p.) inhibited it. P (1 mg/kg) increased the intestinal transit while higher dose (10-20 mg/kg) had no effect. E2 suppresses gastric motility response to thyrotropin-releasing hormone (TRH) and restraint stress in conscious rats [14]. The precise sex steroid that is responsible for these changes is controversial. It appears that sex steroids play an important role in modulating these effects as postmenopausal women being treated with sex hormone-replacement therapy (HRT) had a decreased rate of gastric emptying of solids compared with men. In contrast, postmenopausal women without hormone replacement had rates of solid emptying similar to those of men [24]. Results in pre- and postmenopausal women taking sex HRT showed they had slower gastric emptying than men [25].

Recent studies have also confirmed that HRT is associated with an increased risk of IBS [26]. Stress is known to be an important factor in causing IBS, since it significantly alters bowel functions. Several rodent models of bowel dysfunction caused by restraint stress have been investigated for pharmacological analysis of a stress-related bowel disorder like IBS [12,11,127]. There were similarities between the intestinal effects of wrap restraint stress in rats and IBS in human. Therefore, wrap restraint stress rat is an appropriate animal model to study stress-related intestinal dysfunction. The role of sex hormones in the development of IBS is the subject of ongoing study. Our results indicate that in the restraint stress rats, serum levels of E2 and P were suppressed in OVX rats and restored following chronic treatment with ovarian steroids. Meanwhile, OVX vehicle-treatment significantly increased the number of fecal pellets, more than the time of mucous pellets output. Our study indicated that E2 and P could relieve the rat colon contractile response to restraint stress, thus decrease the number of fecal pellets and increase the number of fecal pellets output.

5-HT3R is expressed by most myenteric neurons, including those that excite gastrointestinal muscle [26-30]. In contrast to other serotonin receptor subtypes, these receptors are ligand-gated ion channels involved in rapid excitatory responses in peripheral and central nervous system [11]. 5-HT3R mediates a fast inward current in myenteric neurons [29,30], and their activation is thought to enhance cholinergic transmission via the release of acetylcholine from parasympathetic nerve terminals [13], thus resulting in an increase in gastrointestinal motility, fluid secretion and pain. Clinically, 5-HT3R antagonists are important in the treatment of symptoms in IBS [12].

Previous studies demonstrated that restraint stress resulted in an increase in fecal pellet output in rats fed the diet, as well as diarrhea in food-deprived rats, which are equally mediated through the endogenous activation of the 5-HT3R [12,14]. Clinically, 5-HT3R antagonist drugs appeared to more effectively diminish bowel pattern disruption in women with IBS as compared to men [11]. Recently, it has been demonstrated that both forskolin and 17-beta-estradiol inhibit the function of 5-HT3R in a noncompetitive manner and that this inhibition is independent of cAMP levels [15]. Because 5-HT3R plays a role in the pathogenesis of IBS, it is possible that the attenuated development of IBS in female rats might be related to the modulation of gonadal hormones on 5-HT3R and GI system. This study focused on the effects of gonadal steroid hormones on 5-HT3R mRNA expression in colon of wrap restraint stress rats. We observed an increase in 5-HT3R gene expression in OVX rats. The increase in 5-HT3R gene expression was similar to that seen in the sham-operated group.
expression was prevented by treating the OVX rats with a combination of E2 and P. These results suggest that 5-HT3R mRNA expression is sensitive to the absence of E2 and P. Their roles in regulation of 5-HT3R mRNA expression deserve further study. If the increase in the density of 5-HT3R gene expression induced by E2 and P deficiency reflects overactivity of 5-HT3R, then such changes may be relevant to hormone- and age-related GI dysfunction.

In conclusion, we suggest that female gonadal hormones may play an important role in regulation of colon 5-HT3R. Exploration of 5-HT3R expression by gonadal steroid hormones could contribute to a better understanding of the interactions between ovarian steroids and GI and may have major pathophysiological implications for 5-HT3R-related bowel disorders, such as IBS.

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