Estradiol upregulates the expression of oxytocin receptor in colon in rats

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Submitted 18 July 2008; accepted in final form 23 February 2009

Estradiol upregulates the expression of oxytocin receptor in colon in rats. Am J Physiol Endocrinol Metab 296: E1059–E1066, 2009. First published March 3, 2009; doi:10.1152/ajpendo.90609.2008.—The study was designed to investigate the effect of estradiol on the excitatory effect of oxytocin (OT) on colon motility. Female Wistar rats were used, and some of them were ovariectomized (OVX) and treated with vehicle or estradiol (E2). A plastic balloon made of condom was inserted into colon to monitor the change of colonic pressure in vivo. Longitudinal muscle strips of distal colon were prepared to monitor the spontaneous contraction of colon in vitro. Expression of OT receptor (OTR) was investigated by Western blot analysis. Expression of OTR mRNA was detected by RT-PCR. Immunohistochemistry was used to locate OTR. In OVX rats, pretreatment of E2 (4–100 μg/kg sc) dose-dependently increased the excitatory effect of OT on colon motility both in vivo and in vitro and increased the expression of OTR and OTR mRNA in colon. Systemic administration of OT excited the colon motility in vivo in rats at periods of proestrus and estrus but did not influence it at diestrus period, when the concentration of plasma E2 was lowest in the estrous cycle. Pretreatment of atosiban, the specific OTR antagonist, and TTX, the blocker of voltage-dependent sodium channel on nerve fiber, attenuated the excitatory effect of OT on colon motility. OTR was located in myenteric plexus of colon. These results suggested that E2 increased the excitatory effect of OT on colon motility by upregulating the expression of OTR in myenteric plexus.

Estradiol (E2) has been traditionally regarded as a hormone that is involved in parturition and milk ejection. In recent years, more evidence has indicated that OT may play a role in regulation of the gastrointestinal (GI) functions such as motility, sensation (13, 18), and immune response to inflammation (4, 10). Exogenous OT influences the GI motility, although the reports are diverse because of the differences of species, methods, and area of the gut (18, 24). It has been suggested that the effect of OT on GI motility might be physiological. OT is released in response to a fatty meal in women (16). Systemic administration of atosiban, the antagonist of OT receptor (OTR), inhibited the spontaneous contraction of gallbladder in rabbits (12) and gastric emptying in humans (16). It has been well established that mRNA for OT and its receptor can be found throughout the human GI tract (15), but the cell types that express OTR were undetectable in human gut by indirect immunofluorescence (19).

In women, colonic dysmotility is very common, and bowel function changes during the reproductive cycle. Although the fluctuation of steroid hormones, especially estradiol (E2) and progesterone, were believed to be the major reason, the mechanism has not been clearly illustrated. Steroid hormones regulate the activity of OTR via both genomic and nongenomic pathways (26). E2 induced the mRNA expression of the OTR and increased the OTR density on the membranes of the uterine smooth muscle and central nervous system (8, 21). The recent study of our group also indicated that the pretreatment of E2 increased the inhibitory effect of OT on the contraction of muscle strips of colon (25). Exogenous OT excites the colonic motility in healthy women (17). As far as we know, there is no report about the effect of estrogen on the protein expression of OTR in rat colon. We hypothesized that cyclic change of estrogen in plasma during estrous cycle might influence the expression of OTR in colon. To test this hypothesis, we monitored the colon motility in female rats at different phases of the estrous cycle or in ovariectomized (OVX) rats treated with different dose of E2 or vehicle. The level of OT and OTR mRNA expressed in colon were compared between OVX rats treated with E2 and vehicle. OTR in OVX rats in colon was located by immunohistochemistry.

MATERIALS AND METHODS

Chemicals. OT acetate salt hydrate, β-estradiol 3-benzoate (E2), and sesame oil were purchased from Sigma (St. Louis, MO). Atosiban was obtained from Ferring (Malmo, Sweden).

The Krebs solution was composed of the following reagents (mmol/l): NaCl 120.6, KCl 5.9, CaCl2 2.5, KH2PO4 1.2, MgCl2 1.2, NaHCO3 15.4, and glucose 11.5. E2 was diluted with sesame oil. OT was dissolved in saline.

Experimental animals. Female Wistar rats, weighing 200–220 g, were purchased from the Animal Center at Shandong University. All experimental procedures were performed according to the guidelines of the animal ethics committee of the Shandong University School of Medicine and were approved by the committee. Ovariectomy was performed under light ether anesthesia. The ovaries were picked out by a forceps through a 1-cm incision made over both flanks of the rat. A ligature was placed below the ovary, and the ovary was removed; then the incision of the muscle and fur were sewed up by aseptic suture line, and finally the wounds were disinfected by iodophors. Two hours after bilateral ovariectomy, 32 female rats were randomly divided into four groups. The rats of the three experimental groups were treated with E2 (4, 25, and 100 μg/kg sc) (5) once daily for 6 days; the rats of the control group were treated with sesame oil (1 ml/kg sc). Twenty hours after the last injection, rats were employed for both experiments.

Female rats at different stages in the estrous cycle were employed, and the stage of each rat was verified by vaginal smears.

Measurement of uterine and E2 concentration. After decapsulation, uteri were removed and weighed. Blood was collected from rats and centrifuged at 3,000 rpm for 30 min. Serum was kept at 4°C.
and then the E2 concentration were measured with an assay under Automated Chemiluminescence System (Roche E170; Roche).

Preparation of muscle strips and recording of the tension of colon in vitro. The muscle strips of colon were prepared as described previously (23). In brief, rats were fasted overnight but allowed to drink water before the experiment. Immediately after rat decapitation, a segment of distal colon (1 cm from cecum) was removed. The segment was opened along the mesenteric border, and the resulting rectangular sheet was pinned flat (mucosa up) in a petri dish filled with oxygenated Krebs solution. Muscle strips (8 × 3 mm) were cut parallel to the longitudinal fibers, and they were designated as longitudinal muscle.

The prepared muscle strips were suspended in tissue chamber containing 5 ml of Krebs solution (37°C), which bubbled continuously with 95% O2 and 5% CO2. One of the narrow ends of the strip was tied to a hook at the bottom of the chamber. The opposing end was connected to an external isometric force transducer (JH-2B; Instrument Company of Chengdu, Chengdu, China). The temperature of the chamber was kept at 37 ± 0.5°C. Tension of colonic strips (under an initial tension of 1 g) in the tissue chambers was recorded using a polygraph system (SMUP-PC; Fudan University, Shanghai, China). To stabilize background contractions after excision, the muscle strips were initially equilibrated in the tissue chamber for at least 90 min. During this period the bath medium (37°C) was replaced every 15 min. OT was administered to the chambers after the spontaneous contraction of colonic strips had become stable. The average muscle tension over 10-s periods was calculated using MF Lab software (Fudan University), which integrated the tension-time trace over each recording period. Each muscle strip was exposed to OT only once, and tension was recorded continuously for 40 min after OT administration.

Recording of the colon motility in vivo. After being anesthetized with amobarbital sodium (0.056 g/kg), the rats were fixed on the homeothermal operation table (37°C) (HM-BXT-3; Huamu Scientific Instruments, Dalian, China), and then rat trachea was cannulated to facilitate the ventilation. To monitor the colonic pressure, a plastic ballonette made of the condom (Sellman; Latex, Tian Jin, China) was inserted into the colon. Another end of the catheter was connected with a pressure transducer (YP-101; Xinhang Xingye Tech, Beijing, China) through the catheter. To monitor the blood pressure (BP), the right femoral artery was cannulated and the catheter connected with the same kind of transducer. The rats with BP <100 mmHg were not employed. The signals from the two transducers were amplified by biological amplifier (ML136; AD Instruments, Sydney, Australia) and recorded by polygraph (Powerlab 8SP; AD Instruments). Normal saline (0.7 ml) was infused into the balloon to maintain the pressure in the balloon at ~10 mmHg. To stabilize the background motility after surgery, the colon was initially equilibrated for 40 min before all experiments were conducted.

Western blot. Muscle strips of the colon prepared as above were homogenized for protein analysis. The homogenates were centrifuged at 2,000 rpm for 10 min at 4°C, and the protein content of the supernatants was evaluated according to Bradford’s method (3) using Protein Quantitative Analysis kit (k3001-BCA; Shenergy Biocolor, Shanghai, China). Supernatants containing 100 μg of protein were diluted in reducing 2× sample buffer and loaded into 12% SDS-PAGE. After separation by SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked for 3 h at room temperature in blocking buffer (5% nonfat dry milk, Tween-Tris-buffered saline), washed in Tween-Tris-buffered saline (0.1% Tween-20, 50 mmol Tris, and 150 mmol NaCl), and incubated overnight with anti-rat OTR IgG (1:400, sc 8102; Santa Cruz Biotechnology) (14) followed by peroxidase-conjugated secondary antibodies (1:20,000). Finally, immunoreactive proteins were revealed using Twinplate Color Scanner (T1200; AGFA, Shenzhen, China). The above protocol of Western blot followed the method described by other groups (6, 22).

RT-PCR. Muscle strips of the colon prepared as above were homogenized in Trizol (Invitrogen, Shanghai, China) for total RNA extraction. RNA reverse transcription was completed using the high-capacity cDNA reverse transcription kit (4368814; Applied Biosystems). The primer pair of OTR (forward: 5′-TGTCGCCACACCTACC-3′; reverse: 3′-CTACCCGCCTGCTCAGA-5′) and β-actin (forward: 5′-TCTAAATGAGCTCGTGG-3′; reverse: 5′-GGAGTTCACTACACAGTTGATG-3′) was used to amplify their homologues from the extracted genomic DNA (Invitrogen). The PCR was conducted at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and then the final step was 72°C for 10 min. The PCR-amplified fragment size of OTR was 186 bp.

Immunohistochemistry. Rats were fasted overnight but allowed to drink water before the experiment. Immediately after rat decapitation, a segment of distal colon was removed and soaked in 4% paraformaldehyde for 12 h. Fixed tissue was rinsed with 100 min and experienced a series of dehydrating, clearing, and immersing in wax. Then the tissue could be sectioned with 4-μm thickness. Sections were then stained using a two-step method. Activity of endogenous peroxidase was blocked with 3% hydrogen peroxide. After three rinses in PBS, 10% normal rabbit serum was applied for 15 min, and then the sections were incubated with primary goat anti-OT receptor antibody (diluted 1:100 in PBS; Santa Cruz Biotechnology) (11, 14) overnight in a humid chamber at 4°C. After the sections were washed, they were incubated with polymer peroxidase anti-goat serum (ZSGB-BIO, Beijing, China) for 30 min at room temperature. After several rinses, peroxidase was revealed by a 3,3′-diaminobenzidine tetrahydrochloride substrate kit (ZSGB-BIO). Negative controls were performed without primary antibody.

Statistical analysis. In the in vitro studies, the mean value of the colon pressure for 10-s periods was recorded over 0–3 min before treatment with OT was taken as the baseline. The average pressure for a 2- to 4-min period after OT treatment was normalized by subtracting the baseline, where the baseline for each experiment was equal to zero. This value was taken as the change in pressure due to each treatment. In the in vivo studies, the mean value of the average tension for 10-s periods recorded over 0–3 min before treatment with OT was taken as the baseline. The average tension for a 2- to 4-min period after drug treatment was normalized to a standardized ratio (R), where the baseline for each experiment was equal to one. This value was taken as the change in motility due to each treatment. In Western blot and PCR analysis, the value of R is the value of the grayscale division between the OVX rats treated with E2 or vehicle.

The data were presented as means ± SE, with n indicating the number of rats. Data were analyzed with SigmaStat 3.5 software (SPSS, Chicago, IL). One-way ANOVA analysis followed by Dunnett’s test was used to analyze the effect of OT on colon motility and the effect of E2 treatment on OTR mRNA and protein expression. P < 0.05 was considered as significant difference.

RESULTS
Effects of OT on colon motility and BP in OVX rats in vivo. In OVX rats, E2 replacement dose-dependently increased the E2 concentration in plasma and uterine weight (Table 1). In OVX rats treated with sesame oil, systemic administration of OT (1.5 μg/kg iv) transiently increased BP but did not influence the motility of colon. In 1–35 min following OT administration, the colonic pressure was not altered [P > 0.05 vs. normal saline (NS), n = 6; Fig. 1B]. But in OVX rats treated with E2, the same dose of OT significantly increased the colon motility (Fig. 1B). The effect of OT was observed immediately after the agent administration, reached the highest level at 1–10 min, and then decreased to the baseline 40 min later (Fig. 1A and B). In the group that received low doses of E2 (4 μg·kg⁻¹·day⁻¹, 6 days), the mean colon pressure increased by
Table 1. Uterine weight of 2-cm length and plasma concentration of E2 in OVX rats treated with different doses of E2 or vehicle

<table>
<thead>
<tr>
<th>Groups (n = 6)</th>
<th>Uterine Weight, mg</th>
<th>Plasma E2 Concentration, pg/ml</th>
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<tbody>
<tr>
<td>OVX + vehicle</td>
<td>34.25 ± 9.50</td>
<td>29.67 ± 3.69</td>
</tr>
<tr>
<td>OVX + E2, 4 μg/kg</td>
<td>129.00 ± 26.27#</td>
<td>89.58 ± 44.80</td>
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<td>OVX + E2, 25 μg/kg</td>
<td>139.75 ± 13.07#</td>
<td>255.60 ± 70.92#</td>
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<tr>
<td>OVX + E2, 100 μg/kg</td>
<td>144.75 ± 16.05#</td>
<td>800.40 ± 210.51#</td>
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Values are means ± SE. E2, estradiol; OVX, ovariectomized. #P < 0.05 vs. vehicle control.

2.10 ± 1.25 mmHg in 1 min (P < 0.05 vs. NS, n = 8) and returned to normal 20 min later (P > 0.05 vs. NS, n = 8; Fig. 1B). Treatment of middle (25 μg·kg⁻¹·day⁻¹, 6 days) and high (100 μg·kg⁻¹·day⁻¹, 6 days) dose of E2 exerted a more profound influence on the excitatory effect of OT on colon motility in vivo (Fig. 1B).

In OVX rat with E2 replacement (25 μg·kg⁻¹·day⁻¹, 6 days), OT (0.6–3 μg/kg iv) dose-dependently increased colonic pressure (Fig. 2A). Following OT (1.5 μg/kg iv) administration, the excitatory effect of OT reached maximum (Fig. 2A), and higher dose of OT (3.0 μg/kg iv) did not further increase the response of colon (Fig. 2A). OT (1.5–3 μg/kg iv) transiently increased BP in OVX rat with E2 replacement (25 μg·kg⁻¹·day⁻¹, 6 days) (Fig. 2B). Following OT administration, BP increased at 1 min and returned to normal 2 min later (Fig. 2B).

Effects of OT on the colon contraction in vivo in female rats at different stages in estrous cycle. The concentration of E2 is highest in rats at proestrus and lowest in diestrus stage (Table 2). Systemic administration of OT (1.5 μg/kg) significa-
cantly increased the colon motility in rats at proestrus and estrus but did not affect colon motility at diestrus stage (Fig. 3). This effect appeared 3 min after OT administration and reached the highest level at 10–15 min. In proestrus rats, the mean colonic pressure increased to 1.50 ± 1.17 mmHg in 10 min after OT administration (P < 0.05 vs. before OT administration; Fig. 3).

Effect of atosiban on the colonic pressure and excitatory effect of OT on colon motility. Pretreatment of atosiban (3.75 μg/kg iv) completely abolished the excitatory effect of OT (1.5 μg/kg iv) on the motility of colons in OVX rats with E2 replacement (Fig. 4). With the treatment of atosiban, the change of mean pressure of colon 1 min after OT administration was -0.11 ± 0.34 mmHg (P < 0.05, n = 5), significantly lower than that of the group treated with the same dose of OT but without pretreatment of atosiban (4.09 ± 1.06 mmHg), which acted as the control. Intravenous injection of atosiban (3.75 μg/kg iv) did not influence the motility of colon (Fig. 4).

Effect of vasopressin on colonic pressure and BP in OVX rats with E2 replacement. To exclude the possibility that OT influenced colonic motility through vasopressin (VP) receptors, we investigated the effect of VP on colon pressure in OVX rats with E2 replacement (25 μg/kg iv) and atosiban (3.75 μg/kg iv) on the motility of colon in OVX rats with E2 replacement (Fig. 4). With the treatment of atosiban, the change of mean pressure of colon 1 min after OT administration was -0.11 ± 0.34 mmHg (P < 0.05, n = 5), significantly lower than that of the group treated with the same dose of OT but without pretreatment of atosiban (4.09 ± 1.06 mmHg), which acted as the control. Intravenous injection of atosiban (3.75 μg/kg iv) did not influence the motility of colon (Fig. 4).

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Table 2. Levels of plasma E2 in cycling female rats at different stages

<table>
<thead>
<tr>
<th>Stage of Estrus Cycle</th>
<th>Plasma E2, pg/ml</th>
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<tr>
<td>Proestrus (n = 6)</td>
<td>58.64±12.66#</td>
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<tr>
<td>Estrus (n = 6)</td>
<td>37.99±16.00</td>
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<tr>
<td>Diestrus (n = 5)</td>
<td>28.75±2.11</td>
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Values are means ± SE. #P < 0.05 vs. diestrus.

Effects of OT on colon contraction in vitro in OVX rats replaced with E2. OT (10^-7 M) excited the motility of longitudinal muscle strips of distal colon of OVX rats treated with middle and high doses of E2 (25 and 100 μg/kg, respectively) but did not influence the contraction of muscle strip of colon in OVX rats treated with oil (vehicle control) and low dose of E2 (Fig. 6, A and B). The muscle contraction appeared small and irregular immediately after OT administration. But 10 min later, there were big and regular contractions (Fig. 6A). In the group that received high dose of E2, R value increased from 0.99 ± 0.22 (baseline) to 1.21 ± 0.11 (n = 7, P < 0.05) at 15 min following OT administration (Fig. 6B).

OTR expression in colon of OVX rats with E2 replacement. E2 replacement significantly upregulated OTR expressed in colon of OVX rats (Fig. 7A). Treatment with middle dose of E2 increased the relative amount of OTR in colon from 1 (vehicle control) to 2.84 ± 1.88 (P < 0.05, n = 6). High dose exerted a similar effect (Fig. 7B).

Expression of OTR mRNA in colon of OVX rats with E2 replacement. E2 replacement significantly upregulated the expression of OTR mRNA in colon of OVX rats. Middle dose of E2 increased the relative amount of OTR mRNA in colon from 1 (vehicle control) to 2.018 ± 0.808 (P < 0.05, n = 6) (Fig. 8).

Location of OTR in colon in OVX rats with E2 replacement and the effect of TTX on the excitatory effect of OT on the contraction of muscle strips. The cells with OTR immunoreactivity were located in myenteric plexus of colon and smooth muscle of uterine smooth muscle (Fig. 9, A–D). No immunoreactive cell was detected if the tissue was treated with the second antibody alone (without the primary antibody; Fig. 9E). E2 replacement (25 and 100 μg/kg, 6 days) increased the number of OTR-immunoreactive cells and the intensity of the immunoreactivity of the labeled cells (Fig. 9, B–D). To further testify the finding that OT was expressed in enteric nervous system, we investigated TTX, the specific blocker of voltage-dependent sodium, on nerve fiber. Pretreatment of TTX (10^-5 M) completely blocked the excitatory effect of OT on the contraction of longitudinal muscle strips of proximal colon.
from OVX rats with E2 replacement (25 μg·kg⁻¹·day⁻¹, 6 days; Fig. 9F).

DISCUSSION

The present study indicates that systemic administration of OT did not affect the colon motility in OVX rats without E2 replacement but increased it significantly when the OVX rats were treated with E2 (4–100 μg). Similar effect of OT was observed in vitro. The same dose of OT excited the colon motility in female rats at proestrus and estrus stages but did not influence that in diestrus stage. VP dose-dependently increased BP but decreased the colonic pressure. E2 replacement significantly increased the amount of OTR and OTR mRNA in colon in OVX rats. OTR was located in myenteric plexus in rat colon. TTX, the specific blocker of voltage-gated sodium channel, completely abolished the excitatory effect of OT on longitudinal muscle strips of colon in OVX rats with E2 replacement.

As far as we know, this is the first study about the effect of E2 on OTR expression in GI tract. In OVX rats, the plasma concentration of E2 and uterine weight is proportional to the dose of E2 treated following ovariectomy, and this result was reasonable and consistent with the report of other groups (5). In female rats, there is a dynamic change in the plasma concentration of estrogen and progesterone during an estrous cycle. Because both of these two hormones might regulate the expression of OTR in gut, ovariectomy is an ideal method to exclude the effect of endogenous progesterone. Using this model, we investigated the effect of E2 on OTR and OTR mRNA expression in gut and the excitatory effect of OT on colon motility.

In both in vivo and in vitro experiments, OT did not influence colon motility of OVX rats without E2 replacement but significantly increased it if the OVX rats were treated with E2. So it seems that the excitatory effect of OT on the colon motility of OVX rats was estrogen dependent. The strongest evidence to support this hypothesis was that E2 replacement...
increased the expression of OTR and mRNA in colon of OVX rats. These data suggested that E2 increased the colon sensitivity to OT by increasing the OTR amount.

The plasma concentration of estrogen has a dynamic change during the estrous cycle. Some have found that gastric emptying is slower in women than in men (7), and sex hormones can affect the GI motility (5). It has been reported that women experience more abdominal symptoms at the beginning of the follicular phase (2). In female rats, the concentration of E2 in plasma is highest during proestrus period and lowest in diestrus period. To investigate the effect of endogenous estrogen on the excitatory effect of OT on colon motility, we compared the effect of OT (1.5 μg/kg) on colon pressure in vivo in female rats at different periods in estrous cycle. We found that OT did not influence the colon motility if the rats were at diestrus period but significantly increased the colon pressure if the rats were at the other stages. Therefore, we believe that, in female rats with normal estrous cycle, the excitatory effect of OT on colon motility is estrogen dependent.

Oxytocin is also a cardiovascular hormone. OTR is expressed in all heart compartments and vasculature (9). In the present experiments, systemic administration of OT transiently increased BP. This result is consistent with the data of Peterson et al. (20).

OT and VP are structurally related cyclic nonapeptides. Their sequences differ in only two of the nine amino acids. All of the currently known VP/OTR subtypes (V1a, V1b, V2; OT) have been cloned from rat and human tissues. The receptor selectivity of OT and VP for their own receptors is not absolute, and significant cross-talk can occur with OT at VP receptors (and vice versa) at higher concentrations (1). To exclude the possibility that OT may exert excitatory effect on colon motility through VP receptors, we investigated the effect of VP on the colon pressure. We found that although VP dose-dependently increased BP, it decreased colon pressure. This effect was contrary to that of OT on colon muscle strips in vitro and colon pressure in vivo. So we believe that OT excites colon motility through OTR in colon.

Although it is reported that OTR mRNA exists on GI tissue in humans (15), according to our knowledge, this was the first time that OTR was found to be located in colon. We found that, in female rats, OTR was expressed in myenteric plexus. TTX, the specific blocker of voltage-dependent sodium channel, completely abolished the excitatory effect of OT on the contraction of colon in vitro. So it is clear that OT exerted its regulatory effect on colon through receptor in myenteric plexus.

In conclusion, we found that E2 upregulated the OTR in rat colon and then increased the colon sensitivity to the excitatory effect of OT on colon motility. OTR was expressed in enteric plexus in rat colon.
Fig. 9. Immunohistochemistry on paraffin sections obtained from uterus and colon of OVX rats replaced with E2 or oil (A–E) and the effect of TTX on the excitatory effect of OT on the muscle strips from OVX rats with E2 replacement (F). Photographs were taken by light microscopy (400-fold), except for D (200-fold). A: OTR immunoreactivity expressed in myenteric plexus (arrow) of OVX rats treated with oil. B: OTR immunoreactivity expressed in myenteric plexus (arrow) of OVX rats treated with 25 μg/kg E2. C: OTR immunoreactivity expressed in myenteric plexus (arrow) of OVX rats treated with 100 μg/kg E2. D: positive control. OTR antigen is expressed on the smooth muscle (arrow) of uterus from OVX rats treated with 25 μg/kg E2. E: negative control. The specimen was treated with PBS instead of primary antibody. It was clear that the type of the OTR-immunoreactive cell in colon was nerve cell, because it was more orbicular and bigger than smooth muscle cell. F: effect of TTX (10^{-5} mol/L) on the contraction of the excitatory effect of OT (10^{-5} mol/L) on the contraction of longitudinal muscle strips of proximal colon in OVX rats replaced with E2 (25 μg/kg). Pretreatment of TTX blocks the excitatory effect of OT on the contraction of colon. #P < 0.05 vs. OT.
GRANTS

This project was supported by the Natural Scientific Foundation of China (No. 30570832) and NCET-06-0581.

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