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Estrogen rapidly modulates mustard oil-induced visceral hypersensitivity in conscious female rats: a role of CREB phosphorylation in spinal dorsal horn neurons

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Lu C-L, Hsieh J-C, Tsauro M-L, Huang Y-H, Wang PS, Wu L-L, Liu P-Y, Chang F-Y, Lee S-D. Estrogen rapidly modulates mustard oil-induced visceral hypersensitivity in conscious female rats: a role of CREB phosphorylation in spinal dorsal horn neurons. *Am J Physiol Gastrointest Liver Physiol* 292: G438–G446, 2007. First published September 14, 2006; doi:10.1152/ajpgi.00210.2006.—This study investigated the effect of sex hormones on mustard oil (MO)-induced visceral hypersensitivity in female rats and analyzed possible involved signaling pathways. Female rats, either intact or ovariectomized (OVX), were prepared for abdominal muscle electromyography in response to colorectal distension after intracolonic instillation of MO. The effect of MO intracolonic sensitization was evaluated in intact rats, OVX rats, and OVX rats pretreated with a single injection of 17 β -estradiol (E), progesterone (P), E+P, or vehicle. cAMP-responsive element-binding protein (CREB) and phosphorylated CREB (pCREB) were detected in the superficial dorsal horn of L₆ and S₁ in MO or mineral oil-treated OVX rats with/without colorectal distension and estrogen replacement. The distal colorectum was removed for histological evaluation of inflammatory severity in MO-treated intact or OVX rats. The MO-treated rats had significantly higher visceromotor reflex than controls (enhanced visceral hypersensitivity), whereas OVX eliminated this hypersensitivity. After a single injection of E or E+P, the rats rapidly restored MO-induced visceral hypersensitivity within 2 h. Estrogen also rapidly induced a dose-dependent increase in pCREB expression in the superficial dorsal horn neurons in MO-treated, but not mineral oil-treated, OVX rats. The present study suggests that estrogen can rapidly modulate visceral hypersensitivity induced by MO intracolonic instillation in conscious female rats, which may involve spinal activation of the cAMP response element-mediated gene induction pathway.

irritable bowel syndrome; spinal cord; cAMP-response element binding protein; gender

VISCERAL PAIN IS A COMMON complaint frequently encountered in the daily practice of gastrointestinal clinics. Studies have shown women are more likely than men to report pain, have lower pain thresholds, and feel pain with greater intensity (6, 43). Some chronic visceral or musculoskeletal pain syndromes, such as irritable bowel syndrome (IBS), fibromyalgia, and temporomandibular disorders, are also female predominant (11, 21, 44). Even in Eastern society, gender differences in IBS have been reported (28). Similarly, female rats exhibit larger

pain reactions than males to thermal stimuli or formalin testing (4, 38).

Both human and animal studies suggest gender differences in pain processing; however, the roles of sex hormones in visceral pain modulation are conflicting. For example, ovariectomized (OVX) rats exhibit similar or reduced visceromotor reflex (VMR) to colorectal distension (CRD) compared with controls (7, 23), whereas visceral pain behaviors are greatly increased in OVX mice upon intracolonic capsaicin stimulation (41). Estrogen did not affect the VMR by uterine cervical distension in OVX rats (40), whereas the response to CRD was increased (23). These results suggest the interaction between sex hormones and visceral pain may be complex and vary among different organs, stimulation protocols, and experimental models.

Visceral hypersensitivity is crucial to the pathogenesis of IBS (13), which are considered to be the consequence of hypersensitivity of the visceral nociceptive pathways, either of the sensory receptors in the central or periphery neurons (3). The role of sex hormones in the modulation of visceral hypersensitivity also has not been well elucidated. Visceral hypersensitivity can be elicited in animals through central or peripheral manipulation. Neonatal maternal separation in rats can lead to visceral hypersensitivity in response to CRD (12), and estrogen was found to play a regulatory role in stress-induced visceral hypersensitivity (7). Peripheral intracolonic administration of mustard oil (MO) (2) also causes acute mucosal inflammation, which leads to enhanced behavioral and reflex responses to CRD. However, the interaction between ovarian hormones and periphery-induced sensitization remains unclear. In addition, increased phosphorylation of cAMP-response element-binding protein (CREB) contributes to central sensitization in somatic pain (22, 32, 34). Estrogen can also rapidly activate signaling pathways associated with CREB-mediated transcriptional regulation in neurons in the central nervous system and dorsal root ganglion (DRG) (1, 37). Thus we attempted to investigate whether 1) sex hormones can regulate the visceral hypersensitivity induced by peripheral sensitization (MO-induced colitis) in conscious female rats and 2) the phosphorylation of CREB in the spinal cord is involved in this hypothesized sex hormone-dependent visceral hypersensitivity.

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ity. Preliminary reports of these findings exist in abstract form (29).

METHODS

Animal preparation and electrode implantation. This study used female Sprague-Dawley rats (220–330 g) (Laboratory Animal Center, National Yang-Ming University, Taipei, Taiwan), and all the experiments were approved by the local ethical committee. They were maintained on a 12:12-h light-dark cycle with standard laboratory chow and tap water administered ad libitum. Under ether anesthesia, electromyogram (EMG) electrodes made from Teflon-coated stainless steel wire (7-strand) (A-M Systems, Carlsborg, WA) were implanted in the rats' abdominal external oblique muscle at least 7 days before experimentation. Electrodes were exteriorized onto the back of the neck.

Rectal distension procedure. Rats were placed in plastic tunnels (6-cm diameter, 25-cm length) for the experiments. In the 3 days preceding the experiments, the rats were trained to stay in the tunnels. The CRD balloon was a latex glove finger (7 cm long) attached to a catheter (disposable rectal catheter for abdominal pressure measurements; 3.5 mm in diameter, 20 cm in length; Medtronic, Skovlunde, Denmark). The balloon was inflated and left overnight to overcome the tension in its wall. The inflatable device was introduced through the anal canal into the rectum in conscious rats and secured to the tail base. The tube was then connected to a barostat (Medtronic, Skovlunde, Denmark). The colon was distended by inflating the balloon to the desired pressures (20, 40, or 60 mmHg) for 10-s intervals with 30-s intervals between distentions. The distentions were repeated four times during each experimental situation with 5-min intervals between each series (35).

OVX procedure. OVX was conducted as previously described (10). Briefly, ovaries were excised by forceps through a 1-cm incision over both flanks while the rat was under light ether anesthesia. A ligature was placed below the ovary, which was then removed. Ovaries in sham-OVX rats were externalized from the abdominal cavity and replaced without being excised. Rats were allowed 1-wk recovery before experiments. Implantation of EMG electrodes was conducted during ovarian surgery in several rat groups.

Vaginal smear. Ovarian steroid status in intact and sham-OVX rats was evaluated with daily vaginal smears to determine estrous stages, i.e., proestrus, estrus, metestrus, and diestrus (9, 10). Smears were conducted each morning for at least two cycles before the start of each experiment.

Experiment protocol. All experiments were performed at the same time of day (between 9:00 AM and 12:00 PM) to minimize circadian rhythm influence. Six series of experiments were performed.

The first series attempted to verify whether MO induces visceral hypersensitivity in conscious female rats. In the experimental group ($n = 8$), each rat underwent two sessions of CRD during the proestrus stage. These two sessions were separated by 4–5 days, corresponding to rat ovarian cycles. During *session 1* in the experimental group ($n = 8$), mineral oil (vehicle, 0.1 ml) was instilled intracolonic 30 min before CRD; in *session 2*, 2.5% MO (0.1 ml) (Sigma-Aldrich, St. Louis, MO) in mineral oil was applied into the rat's rectum. In the control group ($n = 8$), mineral oil was administered during both sessions. The CRD procedure with simultaneous EMG recording was performed as described. The EMG was recorded by using a CED 1401 instrument and analyzed with Spike 2 software for Windows (Cambridge Electronic Design, Cambridge, UK). The raw EMG signal was rectified off-line and the area under the curve (AUC) for baseline activity in each session was subtracted from the AUC for the rectified response to CRD to obtain an AUC difference (30). In each session, the EMG values from individual distensions were averaged.

Experiment 2 investigated the effect of OVX on visceral hypersensitivity. Similar to the protocol used in *experiment 1*, the abdominal muscle responses to CRD of OVX and sham-OVX rats ($n = 8$ in each

group) were recorded after mineral oil (*session 1*) or MO (*session 2*) administration. These two sessions were separated by 4–5 days, and the experiment was conducted in the proestrus stage in sham-OVX rats.

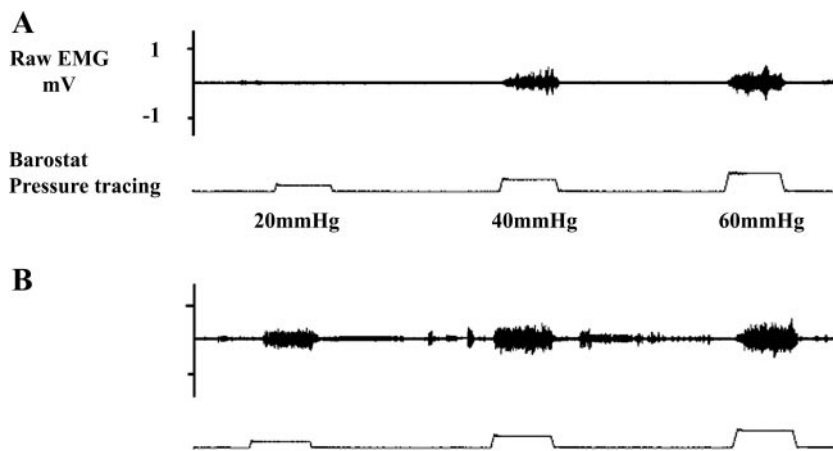
Experiment 3 determined the plasma levels of sex hormones at different time points after a single-dose subcutaneous injection of 17 β -estradiol (E; 10 μ g/kg) or progesterone (P; 20 mg/kg) in OVX rats. This experiment was designed to determine the optimal time point for sex hormones replacement in subsequent experiments. Protocol and dosage were based on data obtained by previous studies (7, 10, 23). Estrogen and progesterone plasma levels after a single injection of either sex hormone were determined in two OVX rat groups ($n = 5$ in each) at 1.5 h, 4 h, 24 h, 48 h, and 5 days. Plasma concentrations of estradiol and progesterone were measured by radioimmunoassay (RIA), as described previously (27, 31). With anti-estradiol serum no. W1, estradiol RIA sensitivity was 3 pg per assay tube. The intra- and interassay coefficients of variability were 7.0% ($n = 5$) and 6.4% ($n = 5$), respectively. With anti-progesterone serum no. W5, progesterone RIA sensitivity was 45 pg per assay tube. The intra- and interassay coefficients of variability were 8.7% ($n = 5$) and 14.0% ($n = 7$), respectively.

Experiment 4 examined the effect of ovarian steroids on visceral sensitivity to CRD. We administered subcutaneous injections of vehicle (sesame oil, $n = 6$), 17 β -estradiol ($n = 8$, 10 μ g/kg), progesterone ($n = 6$, 20 mg/kg), and both 17 β -estradiol and progesterone ($n = 8$) 1 h before mineral oil or MO intracolonic instillation to the OVX rats. In *experiment 3*, sex hormone plasma levels peaked at 1.5 h after the injection and there was a 30-min time lag between MO instillation and CRD. As a result, we chose to administer the hormone injection at 1 h before MO administration. Hormone or vehicle was injected twice, 4 days apart (for each of the two tests), to match the 4-day cycle in intact females. All groups underwent CRD after mineral oil or MO intracolonic instillation in two sessions (separated by 4 days). Our experimental results indicated that MO-induced visceral hypersensitivity is estrogen-dependent.

Experiment 5 was designed to assess the role of CREB in mediating the estrogen-dependent effect on visceral hypersensitivity. One hour after subcutaneous injections of vehicle and 17 β -estradiol (10 or 50 μ g/kg), mineral oil or MO intracolonic instillation was administered to OVX rats ($n = 30$). After 30 min, a 10-min phasic CRD consisting of series of 10-s stimulations to 30 mmHg with 30-s resting intervals between distentions was applied. Rats were then killed to determine the estrogen effects on expressions of CREB and phosphorylated CREB (pCREB) in the spinal cord. For further characterizing the estrogen effect on cAMP response element-mediated gene induction pathway, OVX rats (without CRD stimulation) treated by vehicle and 17 β -estradiol (10 μ g/kg and 50 μ g/kg) ($n = 4$ in each group) were tested for spinal pCREB expression. The effect of CRD alone on the spinal pCREB expression was also tested in OVX rats with phasic CRD and sham stimulation ($n = 4$, in either group).

Under deep anesthesia with pentobarbital sodium (70 mg/kg), animals were perfused transcardially with normal saline followed by 4% paraformaldehyde in ice-cold PBS. The spinal cord L₆ and S₁ segments were excised and postfixed in the same fixative at 4°C for 4 h. All specimens were cryoprotected in 30% (wt/vol) sucrose. Spinal cords were cut transversely at 40- μ m thickness by use of a cryostat. Sections were then collected in PBS. The immunohistochemical study was performed as described previously (19). Briefly, after being washed in Tris-buffered saline [TBS, 25 mM Tris (pH 7.5) and 0.85% NaCl], free-floating sections were treated with 0.2% H₂O₂ until the bubbles disappeared. Nonspecific binding was blocked by 3% normal goat serum plus 2% BSA in TBS containing 0.3% Triton X-100 for 1 h. Primary antibodies applied to floating sections were anti-CREB antibody (1:2,500) (Upstate Biotechnology, Lake Placid, NY) and anti-pCREB antibody (1:1,000) (Upstate Biotechnology). After overnight shaking at 4°C, sections were incubated for 1 h with goat anti-rabbit biotinylated secondary antibody (1:1,000) (Pierce, Rock-

Fig. 1. An example electromyogram (EMG) recording demonstrating the effect of intracolonic mineral oil (A) or mustard oil (MO; B) application on EMG responses evoked by colorectal distension (CRD) in conscious, intact, female rats. Sessions A and B were performed during proestrus phase and separated by 4–5 days. The raw EMG signal increased gradually in a pressure-dependent manner in both sessions, and instillation of MO induced a significantly increased EMG signal in response to CRD for all pressures between 20–60 mmHg.



ford, IL), followed by avidin-biotin-horseradish peroxidase complex (Pierce) for 1 h, with intervening washes with TBS containing 0.3% Triton X-100. Antigen was visualized by combining equal volumes of an ammonium nickel sulfate solution (30 mg/ml in 0.1 M sodium acetate, pH 6.0) in the presence of 0.03% hydrogen peroxide. Following washing and dehydration through a graded series of ethanol solutions, sections were coverslipped with mounting medium Permount (Merck, Darmstadt, Germany). Sections from the three experimental groups (either mineral oil or MO) were processed simultaneously with the same solution.

CREB or pCREB positive cells were counted through the MCID system (Imaging Research, Brock University, St. Catharines, Ontario, Canada) (20). Images were acquired using a charge-coupled device camera mounted on a light microscope at $\times 100$ magnification, and the number of CREB- or pCREB-positive cells in the bilateral superficial dorsal horn was automatically counted by using the “scan area” mode of the MCID imaging system. Fixed sample areas were digitized in five L₆ or S₁ spinal cord sections randomly selected from each rat and quantified for the CREB-immunoreactive (IR) or pCREB-IR cells.

Experiment 6 attempted to determine whether MO-induced colonic inflammation is affected by estrogen or progesterone. Again, intact rats in the proestrus phase were treated with mineral oil in session 1 and mineral oil or MO in session 2 ($n = 5$ in each group). Similarly, four groups of OVX rats preinjected with vehicle (sesame oil), 17 β -estradiol, progesterone, or both 17 β -estradiol and progesterone ($n = 5$ for each group) were treated with intracolonic mineral oil (session 1) and MO (session 2). After MO treatment, the distal 7 cm of the descending colon and rectum in the six groups was removed, placed in 10% formalin, and histologically examined. A cross section of the colon wall was fixed in formalin, dehydrated in graded alcohols and xylene, embedded in paraffin, cut serially into four 50- μ m sections (8 μ m apart) and stained with hematoxylin and eosin. Severity of colon lesions was graded as follows: none = 0, mild = 1, moderate = 2, and severe = 3 (30). Inflammation in the colonic mucosa (none, mild, moderate, or severe) was graded after blind examination by a gastrointestinal pathologist (30).

Statistical analysis. Differences between and among groups were analyzed by Student's *t*-test, one-way ANOVA, and post hoc by Tukey's test or two-way repeated-measures ANOVA and post hoc with paired *t*-test using Bonferroni's correction for multiple comparison. Values are expressed as means \pm SE. Differences were considered significant at $P < 0.05$.

RESULTS

MO-induced visceral hypersensitivity. Figure 1 shows a typical example of MO-induced visceral hypersensitivity. The VMR reflected by the raw EMG signal increased gradually in

a pressure-dependent manner in both sessions 1 (mineral oil) and 2 (MO). Intracolonic instillation of MO, but not mineral oil, significantly increased the EMG signal in the external oblique abdominal muscle in response to CRD relative to baseline signal ($P < 0.01$, repeated-measures two-way ANOVA, Fig. 2).

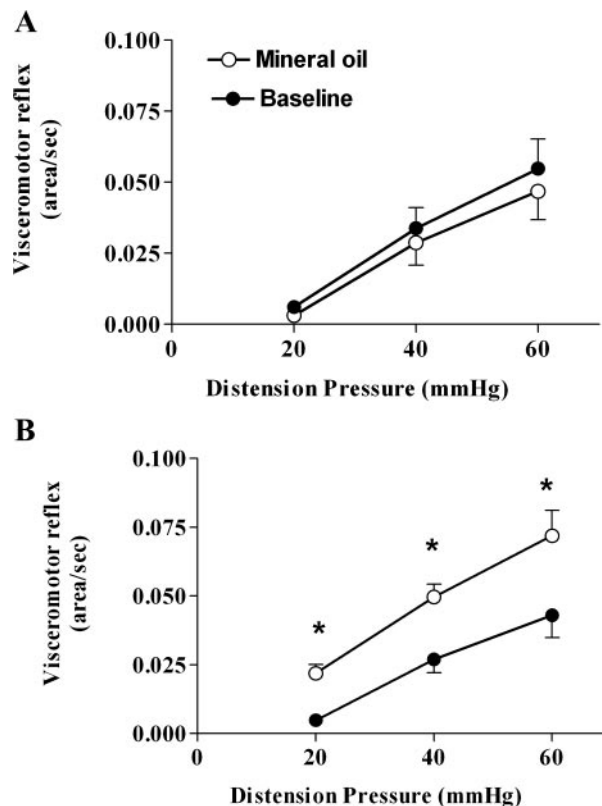


Fig. 2. A: the visceral motor reflex (VMR) after intracolonic mineral oil installation is similar to baseline in conscious and intact female rats. B: on the contrary, the VMR to CRD is significantly higher after intracolonic MO instillation when compared with the baseline (mineral oil instillation) ($P < 0.01$, repeated-measures 2-way ANOVA). The 2 experimental sessions (baseline and MO or mineral oil instillation) were performed during the proestrus phase and were separated by 4–5 days. These results indicate that MO caused visceral hypersensitivity in conscious female rats. *VMR following colonic inflammation is significantly greater than the baseline (paired *t*-test with Bonferroni's correction).

OVX and visceral sensitivity. After MO intracolonic instillation, sham OVX rats had significantly higher VMR (visceral hypersensitivity) than baseline, whereas OVX rats lost the MO-related abdominal muscle hyperresponsiveness to CRD ($P < 0.01$, repeated-measures two-way ANOVA, Fig. 3).

Plasma estrogen and progesterone levels after single injection of sex hormones in OVX rats. In OVX rats, the plasma estrogen level was under the detection limit, and a low baseline progesterone level (15.6 ng/ml) was determined. After a single subcutaneous injection of 17 β -estradiol (10 μ g/kg) or progesterone (20 mg/kg), plasma levels of both hormones increased significantly at 1.5 and 4 h ($P < 0.001$, one-way ANOVA, Fig. 4), returning to preinjection levels by 24 h.

Hormonal treatment and visceral sensitivity. The OVX rats, pretreated with 17 β -estradiol alone ($P < 0.01$, repeated-measures two-way ANOVA) or in combination with progesterone ($P < 0.01$, repeated-measures two-way ANOVA) had significantly higher VMR compared with baseline, whereas the rats with progesterone or vehicle pretreatment showed no evidence of visceral hypersensitivity after MO instillation (Fig. 5). This result suggested that estrogen replacement restored MO-induced visceral hypersensitivity in OVX rats.

Estrogen effects of CREB phosphorylation in spinal dorsal horn neurons. After MO intracolonic instillation, pCREB was rarely observed in the dorsal horn of the OVX rats without estrogen replacement, whereas the immunoreactivity for CREB was markedly expressed. Administration of 17 β -estradiol caused a dose-dependent increase in pCREB expression in

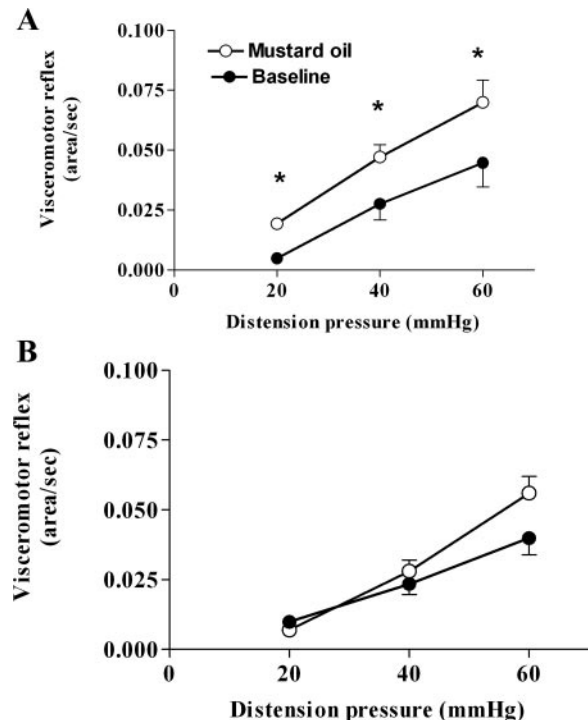


Fig. 3. Effect of MO on ovariectomized (OVX) rats. **A:** the VMR after MO treatment in sham-OVX was significantly higher than that in baseline ($P < 0.01$, repeated-measures 2-way ANOVA). This finding indicates that MO induces visceral hypersensitivity in sham-OVX rats in a manner similar to that in intact rats. **B:** OVX rats did not exhibit abdominal muscle hyperresponsiveness to CRD after MO instillation. *VMR following colonic inflammation is significantly greater than the baseline (paired t -test with Bonferroni's correction).

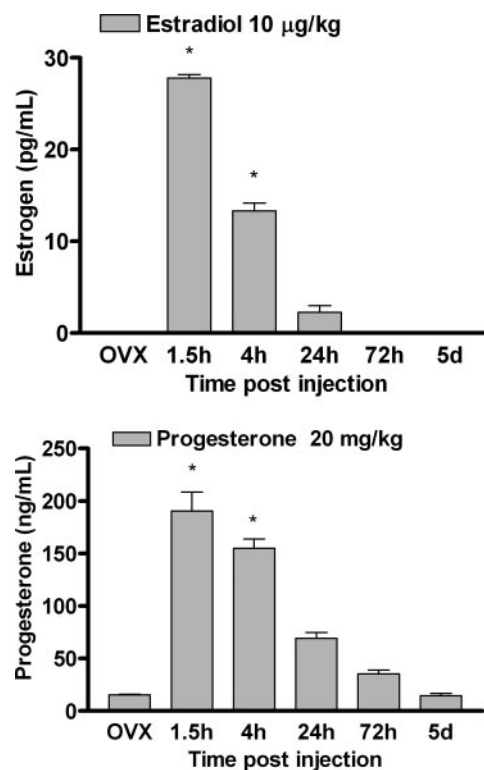


Fig. 4. Plasma estrogen and progesterone concentrations sampled from the OVX rats, at 1.5 h, 4 h, 24 h, 72 h, and 5 days, respectively, after a single injection of 10 μ g/kg 17 β -estradiol (E) or 20 mg/kg progesterone (P) to OVX rats. * $P < 0.001$ compared with OVX or 24 h posthormone injection, 1-way ANOVA and post hoc with Tukey test; $n = 5$ rats in each group.

the superficial dorsal horn neuron in both L₆ and S₁ of the OVX rats that received MO and balloon stimulation ($P < 0.001$, one-way ANOVA, Figs. 6, A and B, and 7). On the other hand, estrogen did not affect the spinal expression of CREB or pCREB expression in the OVX rats with intracolonic mineral oil instillation with CRD ($P = 0.3$, one-way ANOVA, Figs. 6, C and D). Estrogen alone (vehicle vs. 10 μ g/kg vs. 50 μ g/kg: 7.2 ± 1.3 vs. 9.5 ± 1.0 vs. 10.9 ± 1.5 counts/mm², one-way ANOVA, $P = 0.3$) and CRD alone (phasic vs. sham CRD: 10.0 ± 1.0 vs. 7.5 ± 1.5 counts/mm², Student's t -test, $P = 0.1$) did not affect the spinal pCREB expression in OVX rats.

Effects of estrogen and progesterone on colonic inflammation. Histopathological examination demonstrated moderate-to-severe multifocal colonic mucosal or submucosal inflammation among all MO-treated rats, including both the intact and OVX rats with and without sex hormone replacement (Fig. 8). The inflammatory scores were not statistically different between the intact and OVX rats after MO treatment (intact vs. OVX vs. OVX+E vs. OVX+E+P: 2.8 ± 0.2 vs. 2.2 ± 0.2 vs. 2.6 ± 0.2 vs. 2.4 ± 0.2 , one-way ANOVA, $P = 0.3$). Colonic tissues in intact rats treated twice with intracolonic mineral oil had no significant structural damage or loss of crypts (inflammatory score = 0.1 ± 0.0).

DISCUSSION

This study indicated that the visceral hypersensitivity induced by peripheral chemical sensitization (intracolonic MO instillation) is estrogen dependent and may involve spinal

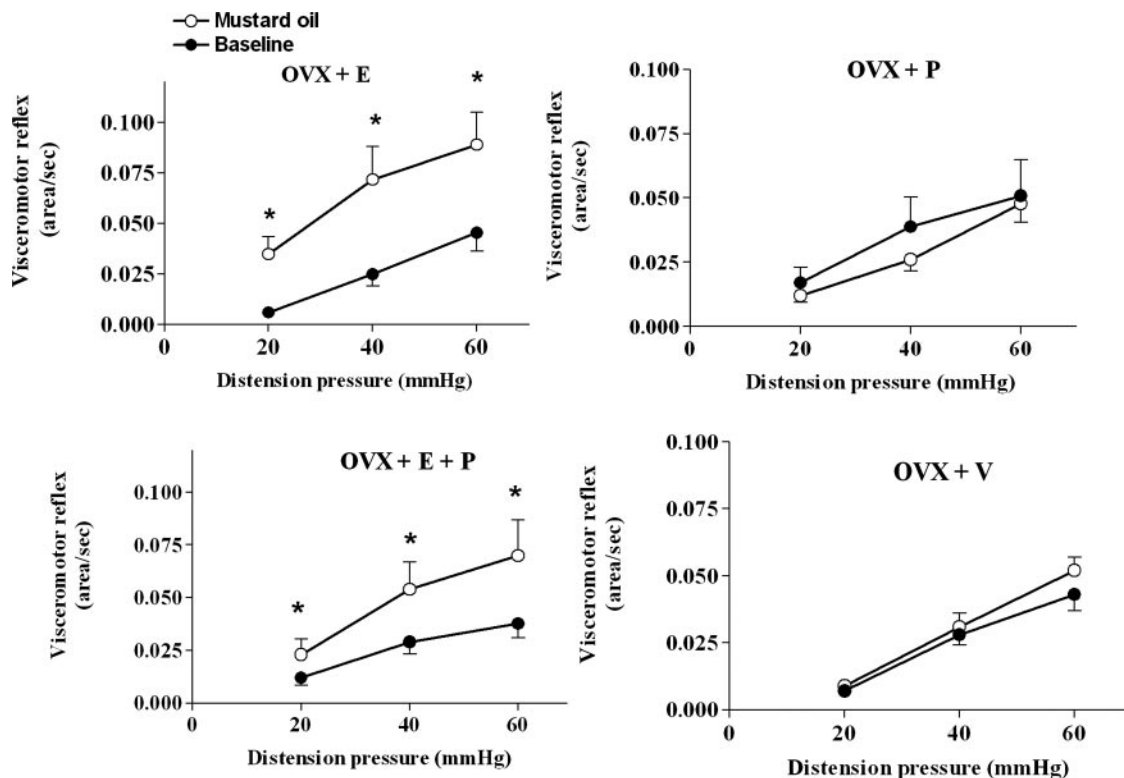
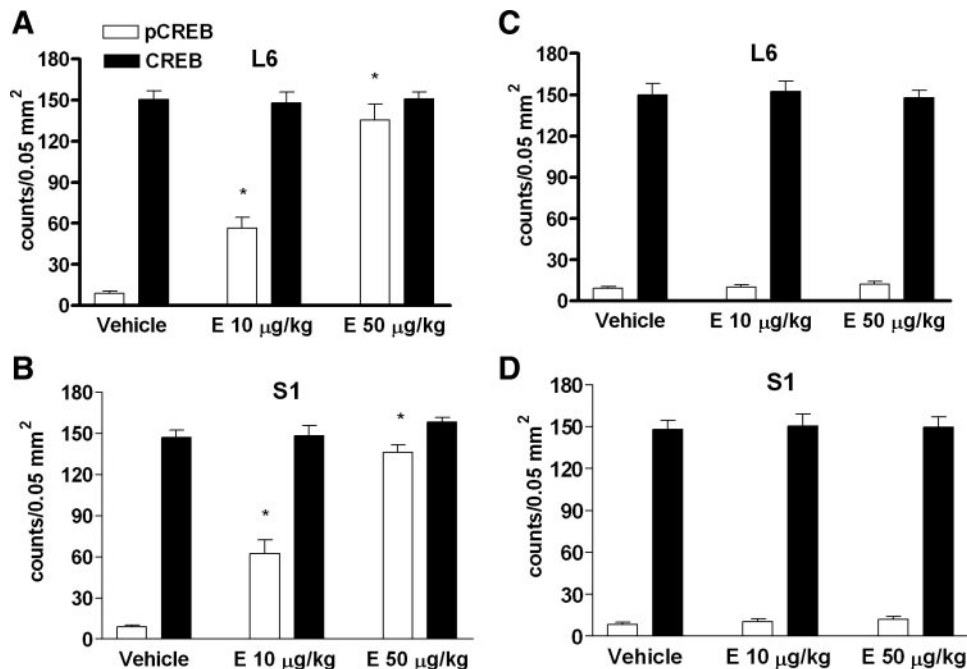


Fig. 5. Effect of pretreatment of OVX rats with E, P, a combination of both E and P (E+P), or vehicle (V, sesame oil) on the abdominal muscle response to CRD after intracolonic mineral oil (baseline) or MO treatment. In 2 h after a single dose injection of estrogen, with ($P < 0.01$, repeated-measures 2-way ANOVA) or without progesterone ($P < 0.01$, repeated-measures 2-way ANOVA), the OVX rats can rapidly restore the MO-induced visceral hypersensitivity. *VMR following colonic inflammation is significantly greater than the baseline (paired *t*-test with Bonferroni's correction).

activation of the cAMP response element-mediated gene induction pathway. This conclusion, in agreement with previous studies, is indicative of the sex hormone's influence on visceral nociceptive processing. Two studies, which used conscious rats as a visceral pain model, have also demonstrated that basal

VMR to CRD or stress-induced visceral hypersensitivity were substantially decreased in conscious OVX rats (7, 23). Another recent study also showed that MO-induced visceral hypersensitivity displayed different pattern between intact and OVX rats (24). All these results suggest that gonadal hormones are

Fig. 6. cAMP-response element binding protein (CREB) and phosphorylated CREB (pCREB) immunoactivity within superficial dorsal horn at L₆ and S₁ level after 10 and 50 $\mu\text{g}/\text{kg}$ 17 β -estradiol compared with vehicle-treated OVX rats after MO (A, B) or mineral oil intracolonic instillation (C, D) and CRD. Estrogen phosphorylated CREB in a dose-dependent manner in the MO-treated group, whereas no effect was noted in the spinal pCREB expression in OVX rats treated with mineral oil and CRD. * $P < 0.001$ compared with vehicle-treated rats (pCREB), by 1-way ANOVA and post hoc with Tukey test; $n = 5$ in each group.



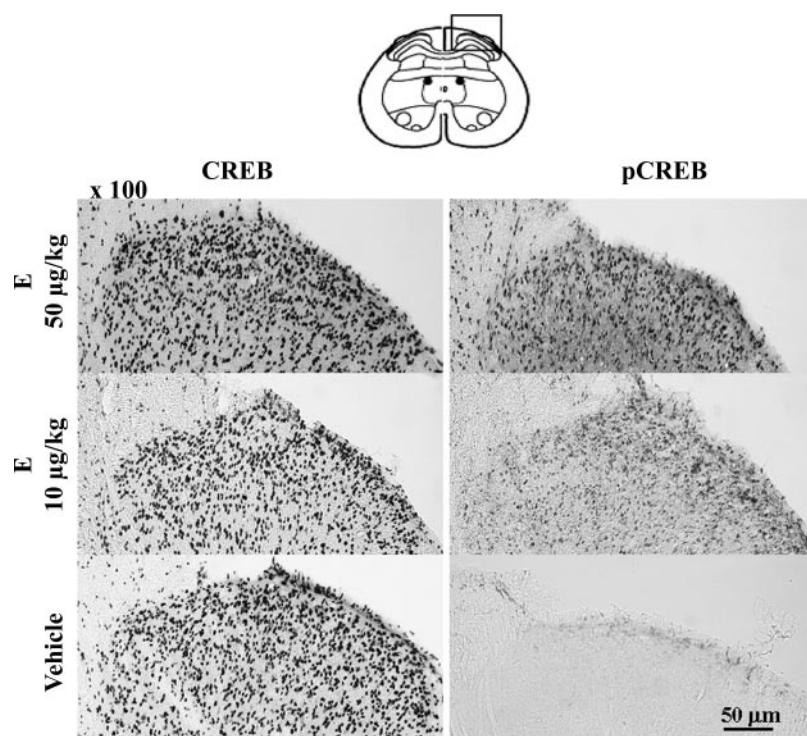


Fig. 7. Example of a single injection of E and vehicle on CREB and pCREB expression in the superficial dorsal horn cells at spinal L₆ level of the OVX rats receiving MO and CRD stimulation. Note the estrogen-induced dose-dependent increase in pCREB-positive cells.

crucial for modulation of viscerosensory processes in conscious female rats.

The effect of gonadal steroids on the nociceptive pathways in visceral pain can act at various levels of the neuraxis, including direct modulation in afferent fiber activity, in the spinal cord, or at the supraspinal level. Two studies have evaluated this mechanism. One demonstrated that estrogen modulates VMR and neuronal activity in the dorsal horn in response to CRD, indicating a critical role for the “spinal cord” in estrogen-mediated VMR (23). The second study demonstrated that estrogen restores stress-induced visceral hypersensitivity in OVX rats, further supporting the role of estrogen and psychogenic factors (“supraspinal” effect) in visceral pain (7). The present study further indicates that visceral hypersensitivity induced by “peripheral chemical stimulation” (MO-induced colitis) is also estrogen dependent. A possible “spinal” mechanism may be involved (see below).

As a stimulus-induced transcription factor, CREB activation requires phosphorylation of the serine-133 residue. Phosphorylation of CREB by protein kinase A or other kinases in the spinal cord has been implicated in pain processing. For example, pCREB levels increase after carrageenan paw inflammation, subcutaneous formalin, and neuropathic pain (22, 32, 34). Increased pCREB levels also correspond to the time frame of hyperalgesia in neuropathic and inflammatory pain (22, 34). Our results demonstrate that the administration of 17 β -estradiol to MO-treated OVX rats resulted in a dose-dependent increase of pCREB expression in the superficial dorsal horn neurons. Thus, activation of the spinal cAMP pathway may be an intracellular mechanism in mediating estrogen-dependent MO-induced visceral hypersensitivity. With respect to the peripheral nervous system, Sohrabji et al. (42) have showed that DRG neurons have estrogen receptors (ERs). In OVX rats, acute estrogen replacement significantly increased preproto-

tachykinin and CGRP mRNA levels in DRG within hours (16, 26). Our previous experiment and others demonstrated that substance P and CGRP are neuropeptides crucial to modulation of visceral hypersensitivity in rats (25, 30, 36). Thus estrogen can also restore MO-induced visceral hypersensitivity via these molecules at the DRG level. Future studies by evaluating the interaction of potential neurotransmitters, spinal pCREB activity, and visceral nociception are mandatory to have a better understanding of the underlying mechanism of the present estrogen-dependent and MO-induced visceral hypersensitivity model.

A recent study used a similar model of MO-induced colitis in OVX rats to investigate the role of sex hormones in visceral hypersensitivity (24). Although they also suggested a modulatory role for estrogen in visceral hyperalgesia, the results between the two studies were quite different. In their study, the MO-induced visceral hypersensitivity could only be demonstrated in high stimulation pressure (60 and 80 mmHg) in intact rats. With or without estrogen replacement, the OVX rats experienced visceral hypersensitivity and the estrogen-replaced OVX rats displayed greater VMR relative to the other groups. On the contrary, we observed a MO-induced visceral hypersensitivity in intact and estrogen-replaced OVX rats, but not in OVX rats. The differences in experimental protocol might explain the discrepancy. First, the estrous phase of their rat was not reported. Since estrous cycle can modulate visceral pain (8, 17), the MO-induced visceral hypersensitivity may vary among different phases of estrous cycle. Second, their experiments were performed on two 4-h sessions in the same day, one with only CRD and the other with CRD after MO instillation. We have already demonstrated that short-term CRD can affect the subsequent VMR response and gene expression in DRG (30). Thus, the VMR in the second session may be affected by the

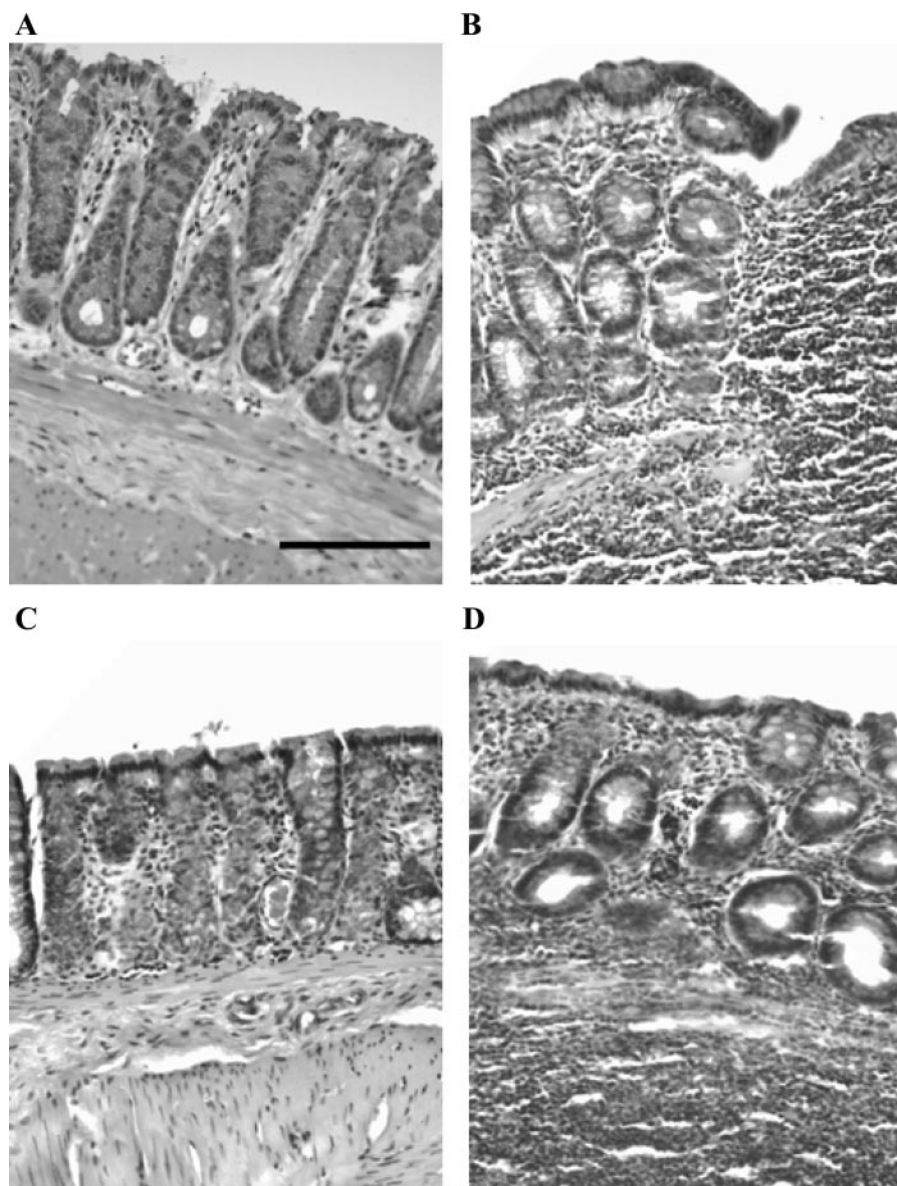


Fig. 8. Histological evaluation of colon tissue (50 μm , hematoxylin and eosin). *A*: the colonic tissue in intact rats with mineral oil intracolonic instillation in both sessions showed intact overlying epithelium without acute inflammation. *B*: with MO application, increased numbers of inflammatory cells were noted in mucosa and submucosa. Foci filled with large amounts of inflammatory cells with damaged crypts and epithelial cells were clearly seen in both intact (*C*) or OVX (*D*) rats with intracolonic MO intracolonic instillation in the 2nd session. Calibration bar equals 100 μm .

CRD in the first session. Furthermore, the long partial restraint time may also affect visceral hypersensitivity in the second session. Third, the sex steroid replacement protocols were different. Both studies applied a single injection for estrogen replacement. However, the CRD was done after 2 days of the injection in Ji's experiment, whereas ours was conducted at 1.5 h postinjection. Since both genomic and nongenomic mechanisms are involved in the action of estrogen (please refer to the discussion below), it is possible that the estrogen may have different effects at different time points. Finally, the lower MO concentration and halothane pretreatment in their study may contribute to an insignificant visceral hypersensitivity at lower stimulation pressure (20 and 40 mmHg). On the other hand, our rats were awake when the balloons were put in place, which may be highly stressful. It is possible that the lack of hyperalgesia in the OVX rats might reflect stress-induced analgesia that is modulated by gonadal hormones (39). Furthermore, only one time point was tested in this experiment (30 min after MO injection), and the hyperalgesia could have

developed in the later period of time after MO injection. Further experiments are necessary to clarify these discrepancies.

Two previous papers had noted that OVX rats displayed a decrease in the baseline VMR when compared with intact rats and the response was restored after estrogen replacement (23, 24). However, we and another study found no such effect (7). The reason for the difference is unclear. However, in Ji et al.'s study (23, 24), brief sedation with halothane was given before CRD, whereas conscious rats were used at the present and Bradesi et al.'s study (7). Whether the anesthetics could contribute to the lowered baseline VMR response in OVX rats deserved further evaluation.

In our study, estrogen restored visceral hypersensitivity in OVX rats in only 2 h. Estrogen affects neurons by binding to ERs, which then bind to estrogen response elements to initiate transcription, i.e., the classic "genomic" mechanism of steroid action. This effect is usually delayed at onset (within hours to days) and prolonged in duration (33). Rapid actions of estrogen

(within seconds to minutes) not involving transcriptional regulation have also been identified (33). These “nongenomic” actions of estrogen have been shown to involve multiple intracellular signaling pathways; however, some still involve ERs (33). For example, a single injection of estrogen can rapidly phosphorylate CREB within gonadotropin-releasing hormone neurons in OVX mice in just 15 min; this activation will persist for 4 h (1). Moreover, estrogen rapidly activates signaling pathways associated with regulation of CREB-mediated transcription in DRG neurons in 10 min (37). It has been reported that an acute blockade of endogenous synthesis of estrogens in quail spinal dorsal horn markedly reduced, within 1 min, behavioral responsiveness to a thermal painful stimuli (14). Additionally, a single estrogen injection restores stress-induced visceral hypersensitivity within 3 h (7). Although the rapid and delayed effects of estrogen are clearly distinguishable from each other mechanistically, gray areas of the uncertainty between rapid- and delayed-onset times exist (33). Further experimentation is required to determine estrogen’s genomic or nongenomic mechanisms mediating this MO-induced visceral hypersensitivity.

The anti-inflammatory activity of estrogen in experimental models has been demonstrated (18). Differences in response to CRD may originate from modulation of colitis by estrogen. That is, the restored MO-induced visceral hypersensitivity in OVX rats may be secondary to diminished inflammation in response to estrogenic effects rather than to estrogen itself. However, a recent study showed that several days and repeated estrogen treatment are required to reverse associated bowel inflammation in a transgenic rat model (18). Our study utilized a single injection of sex hormones and the CRD experiment was performed 30 min following MO instillation. Severity of MO-induced colonic inflammation was similar among groups of intact rats and OVX rats with and without hormonal replacement. A recent study also confirmed that the MO-induced colonic inflammation measured by plasma extravasation is similar with or without estrogen replacement (24). Thus we propose that inflammation per se is, itself, not a factor in mediating estrogen-dependent, MO-induced visceral hypersensitivity.

Concerns may arise over the suitability of a single injection in assessing how estrogen affects the sensory physiology. During the proestrus phase in cycling rats, plasma estrogen concentrations rapidly increase and peak within 8–10 h of entering proestrus. During the following 8–10 h, plasma estrogen decreases as the rat enters the estrous phase (15). In addition to another study, our results demonstrated that a single injection of estrogen induces a rapid increase in blood estrogen levels (23). Thus a single estrogen injection mimics the natural cycle and is suitable for investigating estrogen modulation of pain in rats (5). In fact, two recent studies utilized a single estrogen injection, proving its pivotal role in modulating visceral pain or hypersensitivity (7, 23).

In conclusion, estrogen can rapidly modulate visceral hypersensitivity induced by peripheral chemical sensitization of the colon. Loss of gonadal hormones suppresses the development of visceral hypersensitivity, whereas estrogen replacement results in its restoration. This estrogen-mediated visceral hypersensitivity likely uses spinal activation of the cAMP pathway.

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