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Interactions of estradiol and NSAIDS on carrageenan-induced hyperalgesia

Deirtra A. Hunter^{1,2}, Gordon A. Barr^{2,3}, Kai-Yvonne Shivers¹, Nicole Amador¹, Shirzad Jenab¹, Charles Inturrisi⁴, and Vanya Quinones-Jenab¹

¹Hunter College and The Graduate Center, The City University of New York, New York, 10065

²New York State Psychiatric Institute, New York, New York, 10032

³Children Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

⁴Weill Cornell Medical College, Department of Pharmacology, New York, New York 10065

Abstract

How exogenous estrogen affects inflammatory responses is poorly understood despite the large numbers of women receiving estrogen-alone hormone therapy. The aim of this study was to determine if estradiol alters injury- or inflammation-induced nociceptive responses after carrageenan administration in females and whether its effects are mediated through cyclooxygenase (COX) and prostaglandins (PG). To this end, paw withdrawal latencies and serum levels of PGE₂ and PGD₂ were measured in rats treated with estradiol (0, 10, 20, and 30%) and/or SC 560 (COX-1 inhibitor) or NS398 (COX-2 inhibitor) after intraplantar carrageenan administration. Estradiol significantly increased withdrawal latencies before (baseline condition) and after carrageenan administration. NS398 was anti-nociceptive only in carrageenan treated animals. SC560 increased withdrawal latencies in both paws at 1 and 5 hours after carrageenan administration. Co-administration of estradiol and NS398, but not SC560, were additive except for a prolonged anti-nociceptive effects of estradiol combined with NS398. The anti-nociceptive effect extended beyond that observed with either drug or estradiol alone at the 5-hour time point. Estradiol had no significant effect on PGE₂ serum levels, but both COX antagonists decreased them. Although neither estradiol or the COX inhibitors alone had an effect on PGD₂ serum levels, co-administration of NS398 and estradiol significantly elevated PGD₂ levels. Taken together, our results suggest that estradiol is anti-nociceptive in the thermal test and reduces carrageenan-induced hyperalgesia. These effects are minimally altered through PG-mediated mechanisms.

Keywords

Nociception; carrageenan-test; NSAIDS; ovariectomy; estrogen; sex-differences; prostaglandins; inflammation; thermal nociception

Correspondence: Gordon A. Barr, Department Anesthesiology and Critical Care, Medicine, Children's Hospital of Philadelphia, 3615 Civic Center Blvd., Philadelphia, PA, 19104 267-426-9722 (voice); 267-426-8128 (fax), barrg@email.chop.edu.

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1.0 Introduction

Estradiol has been described as an immunoregulatory agent in that its deprivation increases inflammatory responses whereas its replacement blocks such responses (Ghisletti et al., 2005). Recent studies have shown that estradiol reduces nociceptive responses after an inflammatory stimulus in rats. For example, during Phase II of the formalin test, a behavioral phase associated with inflammatory responses, estradiol attenuates flinching responses, and it does so dose-dependently (Kuba et al., 2005; Kuba and Quinones-Jenab, 2005; Kuba et al., 2006; Mannino et al., 2007). Estradiol replacement also attenuates inflammation and tissue damage associated with paw edema and pleurisy (Cuzzocrea et al., 2000; Cuzzocrea et al., 2001). Aromatase-knockout mice, which lack estrogen production, show increased pain responses after trigeminal formalin administration (Multon et al., 2005). Similarly, estradiol alleviates vaginal hyperalgesia in other persistent/inflammatory pain models (Bradshaw and Berkley, 2002; Tsao et al., 1999).

Estradiol's anti-inflammatory and anti-hyperalgesic effects are receptor mediated. Tamoxifen (an estrogen receptor modulator), but not α -estradiol (an inactive isomer of estradiol) attenuates estradiol's anti-hyperalgesic effects (Kuba and Quinones-Jenab, 2005; Mannino et al., 2007). Sponner et al. (Spooner MF, 2007) and Gardell et al. (Gardell et al., 2008) showed that estradiol's actions are in part mediated through the β -estrogen receptor. Thus, the fact that estrogen receptors mediate estradiol's anti-inflammatory and anti-hyperalgesic responses strongly suggests specificity for estrogen's nociceptive effects.

Prostaglandins (PGs), especially PGE₂, are released at the site of injury and are important mediators of injury-induced nociception (Malmberg et al., 1995; Scheuren et al., 1997; Vetter et al., 2001). Numerous studies show PGE₂ is the dominant PG in spinal cord-mediated nociception and is involved in spinal cord dorsal horn neuronal excitability and synaptic transmission (Ahamadi et al., 2002; Baba et al., 2001; Vasquez et al., 2001). Cyclooxygenases (COX) are the rate-limiting enzymes that catalyzes the conversion of arachidonic acid to PGs (Breder et al., 1995; Tada et al., 2004). The two isomers of COX; COX-1 and COX-2, are differentially activated during inflammatory responses; COX-1 is constitutively expressed, and COX-2 is induced after inflammatory stimuli (Beiche et al., 1996; Breder et al., 1995; Ghilardi et al., 2005; Veiga et al., 2004; Yamamoto and Nozaki-Taguchi, 2002). After injury, levels and activity of COX-2 proteins increase, suggesting a modulatory role for PG in spinal cord sensitization (Adachi et al., 2005; Broom et al., 2004; Durrenberger et al., 2006; Ghilardi et al., 2004). Intrathecally administered NS398 (a selective COX-2 inhibitor) attenuates the level of thermal hyperalgesia in the carrageenan model in a dose-dependent manner, suggesting that spinal COX-2 plays an important role in the maintenance of this thermal hyperalgesia (Yamamoto and Nozaki-Taguchi, 1997). However, although there are abundant data indicating that the inducible isoform, COX-2, is important in inflammation and pain, the constitutively expressed isoform, COX-1, has also been suggested to play a role in inflammatory processes (Smith et al., 1998) although findings remain inconsistent (Burian and Geisslinger, 2005; Tegeder et al., 2001; Whitehouse, 2005; Yaksh et al., 2001).

The carrageenan pain model is a classic inflammatory injury acting through toll-like receptor 4 (Bhattacharyya et al., 2008) to produce a longer-lasting, and more intense inflammation than formalin (Guay et al., 2004; Hargreaves et al., 1988; Ichitani et al., 1997; Nantel et al., 1999). Using this nociceptive model, we aimed to determine first if estradiol attenuates carrageenan induced behavioral responses in female rats and second if so does the mediation occur via COX activity. To this end, carrageenan-induced behavioral responses and PG serum levels were analyzed after SC560 (a selective COX-1 inhibitor) and NS398 (a selective COX-2 inhibitor) in vehicle- and estradiol-treated ovariectomized (OVX) rats.

2.0 Results

2.1 Estradiol/Behavior

The first analysis was the dose effects of estradiol. As expected there were main effects of hormone, paw, intensity and time and but no interactions among them (Figure 1). Because there was no interaction of hormone treatment with the paw ($p = .157$), estradiol was anti-nociceptive both on the injected and contralateral side. Inspection of the figure confirms that. Thus this analysis showed that estradiol reduced nociception in the presence and absence of inflammation. The follow-up analysis was of the carrageenan-injected paw only. The results were similar. Posthoc analysis of the doses showed that the 20% concentration was significantly more effective than either vehicle or 30%; the difference between 10% and 20% concentrations was a trend ($p = .063$) and the 10% did not differ from either the control or the 30% dose. Thus, for the next set of experiments, the 20% concentration was used.

2.2 NS398/Behavior

NS398 significantly elevated withdrawal latencies for both paws ($p < .001$). There was a significant 5-way interaction among all the variables. Figure 2 shows that NS398 was not effective in baseline tests but was after carrageenan injection. Moreover, NS398 was anti-nociceptive with or without estradiol. Analysis of the injected paw data showed a significant hormone by drug interaction ($p = .018$). NS398 was more effective when combined with estradiol. There was also a significant time by NS398 effect ($p < .001$) and a 4-way interaction (Time X Intensity X Drug X Estradiol) in which NS398 had a longer action when combined with estradiol (see 5 hour time point). In the contralateral paw, the effect of NS398 was less than that in the carrageenan injected paw but both estradiol and NS398 increased withdrawal latencies at 5 hours.

2.3 SC560/Behavior

The overall analysis showed a drug effect ($p = .002$), hormone effect ($p < .001$) but no hormone by drug interaction. There were no significant interactions of SC560 with any other variable suggesting that SC560 was anti-nociceptive in both the injected and uninjected paw both under baseline and carrageenan test conditions (Figure 3). When the data from the injected paw were analyzed separately, there was a Time X Drug interaction ($p = .004$); SC560 was more anti-nociceptive after carrageenan injections than under baseline.

2.4 Prostaglandins

Estradiol had no significant effects on either PGE2 or PGD2 (Table 1). NS398 significantly reduced PGE2 levels with or without estradiol and estradiol did not alter the effects of NS398 (Table 2). The effects of NS398 on PGD2 were slightly different; the COX-2 inhibitor enhanced PGD2 levels when combined with estradiol. Neither NS398 nor estradiol had an effect alone. SC560 also reduced PGE2 levels independently of estradiol treatment ($p = .001$) but had no significant effects on PGD2 levels (Table 2).

3.0 Discussion

Previous reports have demonstrated that estradiol attenuates nociceptive responses in inflammatory models (Ozaki-Okayama et al., 2004; Pardutz et al., 2002; Portanova et al., 1996; Taylor et al., 1998; Tegeder et al., 2001). Our data further support these findings and suggest that estradiol's anti-inflammatory effects are not limited to just inflammatory assays but extend to nociception in the absence of inflammation. Specifically, estradiol was anti-hyperalgesic after carrageenan administration, supporting its role in mediation of inflammatory pain, and at the 20% dose increased paw withdrawal latencies (PWL) to a thermal stimulus in the absence of inflammation, before carrageenan administration and in

the contralateral paw. Thus in addition to estradiol's attenuation of injury- induced inflammatory responses, the results show that estradiol's effect on nociception can occur under normal conditions as has been reported before (Li et al., 2009).

The peak effects of estradiol were at the 20% concentration. The 30% concentration was ineffective suggesting that at higher levels estradiol's analgesic effects may cease or that pronociceptive effects may become evident. These findings are in line with other studies that have shown that estradiol can occupy a pro-nociceptive role in humans and rodents (Aloisi and Bonifazi, 2005; Ceccarelli et al., 2002; Dao and LeResche, 2000; Hellstrom and Anderberg, 2003; Kuba et al., 2006; Liu and Gintzler, 2000; Rezaii and Ernberg; Wu et al.). Based on these studies, the anti-nociceptive effects of estradiol are complex and involve a number of variables such as specific pain assay, fluctuating endogenous estrogen/estradiol levels, method of estradiol administration and length of exposure to hormone prior to noxious exposure may contribute to differential effects of estradiol on nociceptive processes.

Similar to previously reported effects in male rats (Yamamoto and Nozaki-Taguchi, 1997), the present study showed NS398 attenuated thermal hyperalgesia in female rats, suggesting that COX-2 plays an important role in the maintenance of induced thermal hyperalgesia by intraplantar carrageenan injection. A novel finding was co-administration of estradiol + NS398 extended the duration of this anti-hyperalgesic effect, longer than that of hormone or drug alone. Moreover, in the contralateral paw 5 hours after carrageenan administration, NS398 was analgesic following carrageenan to the other paw. This might be reflective of later developing central sensitization and may reflect prolonged suppression of cytokine activity by COX-2 inhibition in estradiol treated rats. Although the role of COX-1 in formalin- and carrageenan-induced nociceptive responses remains controversial in male rats (Burian and Geisslinger, 2005; Tegeder et al., 2001; Whitehouse, 2005) in the female rats studied here, antagonism of COX-1 increased the withdrawal latency in the ipsilateral paw after carrageenan. However, the extended anti-hyperalgesic effects observed when NS398 was co-administered with estradiol were absent when combined with SC560.

Carrageenan produces inflammation at the paw and in other peripheral tissues (D'Agostino et al., 2009; Morris, 2003; Willoughby et al., 2000). That estradiol and both COX inhibitors reduced inflammation induced hyperalgesic responses on the ipsilateral paw suggests that both treatments reduce peripheral inflammatory mediators to alleviate nociceptive responses. Estradiol and NS398 but not SC560 reduced paw withdrawal latencies in the contralateral paw. This suggests that NS398 may act to reduce central sensitization. However, the fact that there were no interactions between either COX inhibitor and estradiol does not support a hypothesized COX- PG mechanism for estradiol.

A possible pathway by which estradiol could additionally mediate inflammatory responses would be through activation of cytokine inflammatory mediators, an inflammation-mediated mechanism independent of COX activation. In animal models following trauma-hemorrhage or in experimental autoimmune encephalomyelitis, and in adipose and cardiac tissues, estradiol attenuates levels of pro-inflammatory cytokines, including IL-6 and TNF- α (Bruun et al., 2003; Morales et al., 2006; Suzuki et al., 2007; Xu et al., 2006). Estradiol also reduces the severity of autonomic dysreflexis (an autonomic behavioral condition that manifests after spinal cord injury) and pain in a temporomandibular joint model through reduction of pro-inflammatory cytokines (Guan et al., 2005; Vegeto et al., 2001; Webb et al., 2006). Estradiol's attenuation of pro- inflammatory cytokines has also been reported in humans; for example, plasma levels of these cytokines, including TNF- α and IL-1 β are higher in postmenopausal than in premenopausal women. Furthermore, in postmenopausal women, treatment with physiological concentrations of estradiol significantly decreased IL-6, TNF- α and IL-1 β levels (Berg et al., 2002; Rogers and Eastell, 2001; Uemura et al., 2005).

Estrogen's attenuation of pro-inflammatory cytokine expression in the CNS, coupled with the fact estrogen has a neuroprotective role in many neurodegenerative CNS diseases (such as Alzheimer's and Parkinson's diseases; reviewed in (Czlonkowska et al., 2006)), suggests an important hormone-cytokine link in inflammation. Yet to be elucidated is the extent to which estradiol's regulation of cytokines and/or HPA axis coupled with activation of the COX-2- PG responses mediates the enhanced effect of estradiol and COX inhibition on inflammatory responses.

In the present study, although estradiol lowered PGE2 serum levels, the change failed to reach statistical significance and the dose response pattern differed from that for the behavioral assays. This lack of estradiol effect is consistent with results from Tenenbaum et al. (2007) showing that estradiol did not alter PGE2 levels in neuronal cultures. Both COX inhibitors reduced PGE2 serum levels as compared with control groups. Increases in PGE2 serum levels have been linked to carrageenan-induced behavioral responses and to hyperalgesic states (Durrenberger et al., 2006; Euchenhofer et al., 1998; Haskell, 2003; Hofacker et al., 2005; Tegeder et al., 2001; Vaccarino and Chorney, 1994; Veiga et al., 2004; Willoughby et al., 2000). Thus, the increased paw withdrawal latencies after inhibition of COX enzymatic activities, which in turn lowered PGE2 serum levels, are consistent with previous observations (Pham-Marcou et al., 2008).

It has been postulated that at the spinal cord level PGD2 blocks the PGE2-evoked pain responses, implying that endogenous PGD2 may play an inhibitory role in the appearance of spinal cord nociception under physiological conditions (Minami et al., 1996). NS398 and SC560 alone had no effect on PGD2 levels. In contrast NS398 + EB showed an increase in PGD2 levels that corresponded to the anti-hyperalgesic effect of this combination of drugs. However the effects of this combined treatment on PGD2 did not differ from the effects of estradiol or NS398 alone on PGD2 levels. Although one study showed that estradiol treatment of OVX rats decreased the synthesis and release of PGD2 in uterine tissue (Chaud et al., 1994) after formalin administration to the hindpaw, estradiol administration alone or in combination with the COX inhibitors did not alter PGD2 serum levels in vivo (Hunter et al., 2010). These findings suggest estradiol's anti-hyperalgesic responses are not mediated through changes in PGD2 levels.

The use of hormone replacement therapy (HRT) remains controversial and a task force has recently recommended against its use for the prevention of chronic diseases in postmenopausal women. Today anywhere from 20% to 45% of U. S. women take some form of hormone therapy between the ages of 50 and 75 (NIH. gov). According to industry estimates, about 8 million U. S. women use estrogen-alone replacement therapy and about 20% of hormone users continue for more than 5 years (NIH. gov). Furthermore, HRT is not confined to postmenopausal women, but is used in hypoestrogenic adolescent and young adult females. On the basis of our study, we postulate that hormone replacement may mitigate pain responsiveness and inflammation in women at moderate doses. Because of the importance of this clinical issue, further studies are needed to determine which inflammatory mechanisms, if any, estrogen may modulate to reduce inflammation.

4.0 Methods

4.1 Animals

Eight-week-old OVX female Sprague-Dawley rats purchased from Taconic (Germantown, NY) were double-housed in 12-h light-dark cycle (lights on 8 a.m.) with food and water ad libitum. Animals were randomly assigned to experimental groups. At the time of nociceptive testing, rats weighed between 200 and 240 g. Each study consisted of at least four different cohorts of rats. Animal care was in accordance with the Guide for the Care and Use of

Laboratory Animals (NIH publication 85-23, Bethesda, MD) and approved by the Institutional Animal Care and Use Committee at Hunter College of The City University of New York.

4.2 Reagents

17- β -estradiol 3-benzoate, cholesterol and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). SC560 and NS398 were purchased from Cayman Chemical (Ann Arbor, MI). Carrageenan was purchased from Fluka BioChemika (Ronkonkoma, NY). Sodium chloride solution (0.09%) was purchased from B. Braun Medical Inc. (Irvine CA). Cholesterol was the vehicle of estradiol; DMSO was the vehicle for all other drugs.

4.3 Estradiol replacement

Two weeks after ovariectomy SILASTIC capsules (1 cm, 0.058 in. ID $\times$ 0.077 in. OD, Dow Corning) were inserted into the nape of the animal's neck. Capsules contained either vehicle (100% cholesterol) or estradiol (20% 17- β -estradiol: 80% cholesterol). This dose was chosen because it falls within the range of serum levels during the reproductive cycle and represents the maximally effective dose for attenuating formalin responses without affecting basal activity (Mannino et al., 2007). Moreover, this dose also has been shown to produce persistent anti-hyperalgesic effects after formalin administration (Kuba and Quinones-Jenab, 2005; Kuba et al., 2006; Mannino et al., 2007). Rats were tested 1 week after capsule implantation, a time frame previously shown to produce stable levels of estradiol (Mannino et al., 2006; Mannino et al., 2007).

4.4 Nociception testing

The Hargreaves test apparatus (paw thermal stimulator; University of California, San Diego) consisted of six Plexiglas enclosures (21/8. 5 cm) positioned on a heated glass surface that maintains a temperature of $30^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$. A mobile infrared heat lamp focused one of three heat intensities: Low (4. 50mv); Medium (4. 80mv); and High (5. 20mv), which produced baseline latencies of 15, 10 and 5 seconds respectively. Behavioral tests were conducted between 9:00 a. m. and 3:00 p. m.

Behavioral testing was done 1 week after hormone replacement. To minimize novelty of the new testing environment, animals were placed in the testing chamber for 30 minutes. After this acclimation period, baseline paw withdrawal latencies to the three heat intensities stimuli were determined. Immediately following baseline measurements animals received an i.p. injection of SC560, NS398 or vehicle (20mg/kg). Forty five minutes after this injection rats received an intraplantar injection of carrageenan (100 μ l; 1%) in the right hind paw. Rats were then placed back in the testing chamber and tested at 1 and 5 hours after carrageenan injection. Thermal hyperalgesia was measured via paw withdrawal latency to a focused light source applied to the plantar surface of the injected or contralateral paw. The latency to withdrawal of the paw was automatically recorded. Rats were free to remove their paw at the point of discomfort. Paw withdrawal latencies after carrageenan injection were measured for all three different heat intensities at 1 and 5 hours after carrageenan injection in the right and left paw. A 20 second cutoff prevented tissue damage.

4.5 Prostaglandin measurements

Five hours after carrageenan administration, immediately after the last behavioral test, rats were sacrificed by decapitation (following a brief exposure of 20 s to CO₂), and trunk blood was collected and centrifuged (at 3,000 RPM for 30 min at 4°C). Serum stored at -80°C for 30–60 days until assayed. PGE₂ and PGD₂ serum levels were detected by using enzyme

immunoassay kits from Cayman Chemical (Ann Arbor, MI). PGE2 and PGD2 serum levels are expressed as pg/mL.

4.6 Statistical analysis

For behavioral analysis, data are presented as mean latency of paw withdrawal $\pm$ SEM. The initial analysis was a factorial ANOVA with the between subject variables being: hormone (estradiol or cholesterol) and drug (vehicle vs. NS398 or vehicle vs. SC560). Within subject variables were: paw (ipsilateral or contralateral to injection); time (baseline, 1 hour, 5 hours post-injection); and intensity (low, medium, high). The analysis of estradiol's dose response effects did not include a drug effect. A secondary analysis then focused on the injected paw only, with the same variables. Posthoc tests of the between subjects effects were by Tukey HSD. For serum analysis of PGs, data are presented as mean pg/ml of serum $\pm$ SEM. ANOVAs (variables: Hormone status and/or Drug treatments) were used to determine significant differences in the serum measurements. For all analyses, significance was at the level of $p < 0.05$. For clarity of presentation, the behavioral data are collapsed over the three intensities.

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Figure 1.

The bars for all figures are means $\pm$ one SEM. Estradiol increased the latency to withdraw the paw under baseline conditions, prior to carrageenin injection, in both the injected and non-injected paw. Therefore, although the interactions are plotted, only the main effects of hormone, paw and time were significant. (no significant interaction of paw and hormone). The 10–20% estradiol concentrations were most effective and the higher 30% concentration was not anti-nociceptive, in agreement with prior work.

Figure 2.

NS398 was anti-nociceptive only following carrageenan injection in both paws. There was a significant 5-way interaction among all the variables and a significant 4-way interaction when only the injected paw data were analyzed. Overall the combination of estradiol and NS398 had an effect greater than NS398 alone. At 5 hours the combination had a particularly pronounced effect. There was an anti-nociceptive effect of NS398 in the contralateral paw at 5 hours post-carrageenan.

Figure 3.

SC560 had a small but significant anti-nociceptive effect in both paws under all conditions. There were no significant interactions of SC560 with estradiol. Thus, although the full interactions are plotted here, only the main effects of hormone and drug were significant.

Table 1

Effects of estradiol on prostaglandins

<i>PGE2(pg/ml)</i>			
Chol	10%	20%	30%
137. 2±20.4	89. 7±19.1	134. 5±37.6	114. 3±28.6

<i>PGD2(pg/ml)</i>			
Chol	10%	20%	30%
3944. 5±427.3	4070. 2±282.9	4243. 9±171.4	3307. 2±503.3

Note: Entries are mean values ± one SEM. There were no significant effects of any concentration of estradiol on PGE2 or PGD2.

Table 2

Effects of treatments on prostaglandins

Condition	PGE2(pg/ml)	PGD2 (pg/ml)
Veh/Veh	177. 9±42.5	1813.0±362.8
EB/Veh	120. 4±16.9	2351.0±148.4
Veh/NS398	*74. 8±15.6	2526.1±232.8
EB/NS398	*51. 9±11.5	*3082.7±294.9
Veh/Veh	177. 9±42.5	2512.2± 158.7
EB/Veh	120. 4±16.9	4121.6±1105.7
Veh/SC560	*33. 0± 4.4	2629.7± 189.0
EB/SC560	*60. 2±16.6	3487.5± 514.5

Note: Entries are mean values ± one SEM. Asterisks denote statistical significance from Veh/Veh. The combination of EB/NS398 elevated PGD2 levels compared to control. NS398 and SC560 reduced PGE2 levels with or without estradiol. Note that because PGE2 assays for NS398 and SC560 were run together, the control values are the same.