

ACUTE AND CHRONIC ESTRADIOL REPLACEMENTS DIFFERENTIALLY ALTER CORTICOSTERONE AND COX-MEDIATED RESPONSES TO AN INFLAMMATORY STIMULUS IN FEMALE RATS

Introduction: This study aimed to determine whether the previously reported differential effects of estradiol on inflammation-induced behavioral responses are in part explained through differential activation of the corticosterone-cyclooxygenase (CORT-COX) regulatory pathway.

Methods: Prostaglandin E₂ (PGE₂), COX, and CORT levels were analyzed before and after a formalin administration (1% vs. 5%, representing different intensities of inflammatory stimuli).

Results: In vehicle-treated rats, chronic estradiol administration increased corticosterone, and decreased COX and PGE₂. After acute estradiol administration, although corticosterone serum levels were increased, COX protein levels were unchanged. In rats treated with formalin, PGE₂ serum levels were higher in rats administered 5% formalin than vehicle- and 1%-treated rats. Significant correlations were observed between PGE₂ serum levels, CORT serum levels, and COX protein levels.

Conclusions: Our results suggest that the administration of exogenous estradiol may mediate inflammatory responses by regulating the levels of PGE₂ and/or CORT release, thereby mediating the nociceptive response to an inflammatory stimulus. (*Ethn Dis.* 2010;20[Suppl 1]:S1-50–S1-54)

Key Words: Cyclooxygenase, COX-1, COX-2, Pain, Inflammation, Formalin

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INTRODUCTION

Sex differences in inflammatory pain (reviewed)^{1,2} have been attributed to the effects of estradiol, which has been shown to attenuate formalin-induced behavioral responses dose-dependently.^{3–6} Whereas administration of estradiol via SILASTIC capsules attenuated 5%, but not 1% formalin-induced behavioral responses during Phase II of formalin induced behavioral responses, its administration by subcutaneous injection altered the effects of 1% but not 5% formalin administration.^{4–6} It has been previously postulated that lower concentrations of formalin rely on central sensitization, but higher concentrations rely on both central and peripheral inflammatory mechanisms.⁷ Estradiol's effects have been explained by differential effects of chronic and acute replacement on peripheral inflammation and central sensitization, respectively.^{4,5}

Prostaglandin E₂ (PGE₂) is an important mediator of inflammatory responses after nociceptive stimuli.⁸ Cyclooxygenase (COX), the rate-limiting enzyme involved in PG synthesis, has two isomers – COX-1 and COX-2.⁸ Whereas COX-1 is constitutively expressed, COX-2 is induced after an inflammatory stimulus.⁸ Corticosterone (CORT) has been shown to exert anti-inflammatory effects by negatively regulating COX-1 and COX-2 and decreasing PGE₂ release.⁹ Estradiol decreases PG synthesis in non-central nervous system tissue without altering COX-1 and COX-2 protein levels.¹⁰ This study aimed to determine whether the previously reported differential effects of estradiol replacements in formalin-induced behavioral responses are

in part explained through differential activation of the CORT-COX regulatory pathway.

METHODS

Rats

Eight-week-old ovariectomized Sprague-Dawley rats (Taconic, Germantown, NY) were double-housed under a 12-hr light/12-hr dark cycle (lights on at 8:00 am) with food and water available *ad libitum*. Rats receiving either estradiol or vehicle replacement paradigms were divided into three groups ($n=10$ per group): vehicle, 1% formalin, or 5% formalin administration. One hour after formalin administration, rats were sacrificed by decapitation, following a brief exposure to CO₂ (20 seconds). Behavioral data from these animals have been previously published.^{4,5} Trunk blood and the lumbosacral region of the spinal cord were collected. Animal care was in accordance with the Guide for the Care and Use of Laboratory Rats (Bethesda, MD) and approved by the Institutional Animal Care and Use Committee at Hunter College.

Estradiol Replacement

Chronic Replacement

Two weeks after ovariectomy, SILASTIC capsules (1 cm, .058 inches ID×.077 in. OD, Dow Corning) containing either vehicle (100% cholesterol) or estradiol (20% 17- β -estradiol 3-benzoate: 80% cholesterol) were inserted into the nape of the animal's neck. Formalin testing was done 1 week after surgery.

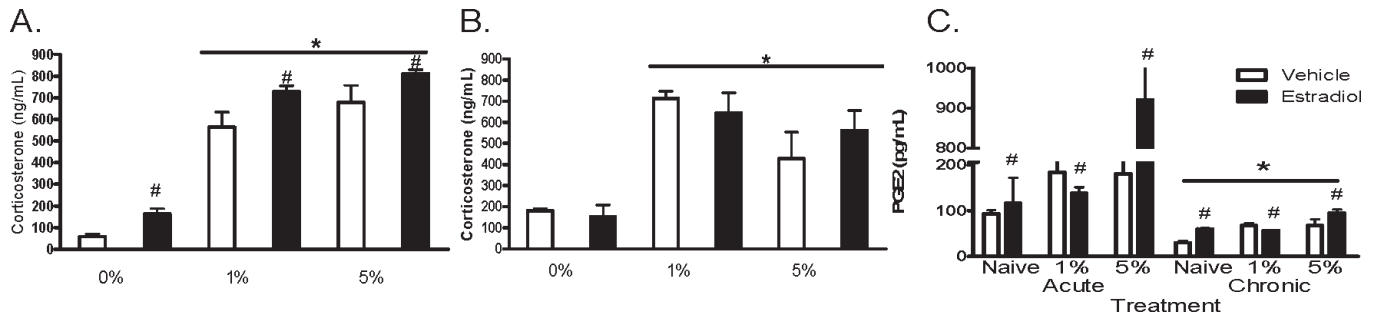


Fig 1. Effects of chronic (A) or acute (B) estradiol replacement on corticosterone or PGE2 (C) serum levels in rats receiving no formalin (vehicle) or 1% or 5% formalin * Indicates a significant main effect of formalin administration. # Indicates a significant main effect of estradiol treatment

Acute Replacement

Forty-eight hours before behavioral testing rats received subcutaneous injections of either estradiol (20 µg) or vehicle (sesame oil). Doses and manners of estradiol administration have been shown to result in levels that fall within the range of serum levels during the reproductive cycle,⁶ and to attenuate nociceptive responses after formalin administration.^{4,5,11}

Radioimmunoassay and Enzyme Immunoassay

Serum was collected after centrifuging trunk blood (3,000 RPM for 30 minutes). CORT serum levels were detected using Coat-A-Count radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA). PGE2

serum levels were detected by using enzyme immunoassay kits from Cayman Chemical (Ann Arbor, MI). Hormone levels were determined by means of a log-logit analysis within GraphPad Prism Software (San Diego, CA).

Western Blots

Protein levels of COX-1 and COX-2 in the spinal cord were analyzed using Western blots as previously described.⁴ Briefly, 30 µg of protein samples were boiled in 1% β-mercaptoethanol-Laemmli sample buffer, separated in gradient SDS-PAGE gels (4%–15%), and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk (30 minutes) and incubated with COX-1 or COX-2 antibodies (1:1000; Cayman Chemical,

Ann Arbor, MI) for 1 hour at room temperature or overnight at 4°C, respectively. After washing in TBST, membranes were incubated with appropriate secondary antibodies (1 hour, room temperature). Band intensities were detected with an enhanced chemiluminescence kit from Amersham (Piscataway, NJ) and quantified with a Molecular Dynamic Computer Densitometer and Image Quant program. Membranes were re probed with α-tubulin antibody (1:1000; Sigma Aldrich, Saint Louis, MO) to normalize band intensity.

Statistics

For each estradiol treatment (acute and chronic), two-way ANOVAs were done (formalin [vehicle, 1%, or

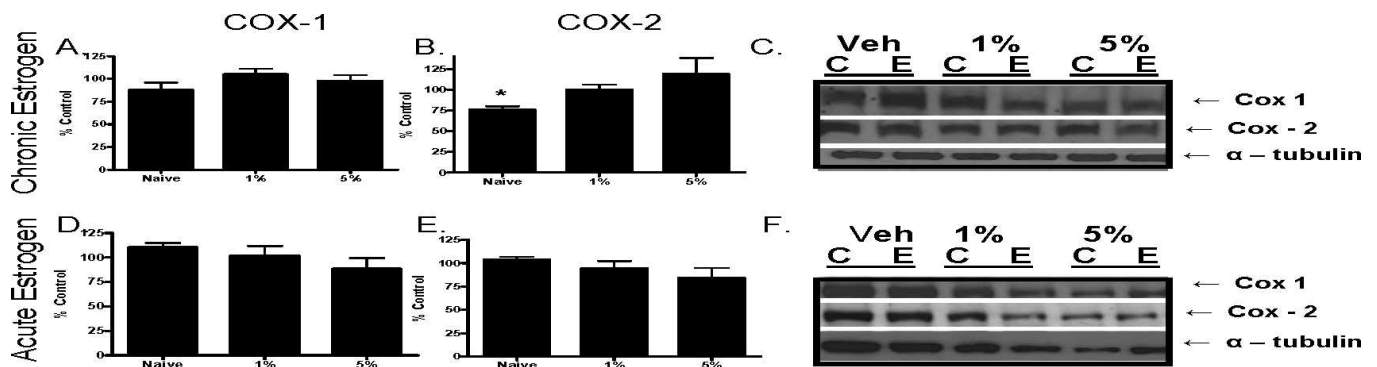


Fig 2. Effects of chronic (A to C) and acute (D to F) estradiol replacement on COX-1 (A and D) and COX-2 protein (B and E) levels. Protein level is expressed as a percentage of its vehicle-treated counterpart group. Representative Western blots for COX-1 and COX-2 are shown (C and F). * Indicates a significant difference as compared with vehicle-treated rats. C: Hormone-vehicle; E: Estradiol; Veh: vehicle

Table 1. Correlation of corticosterone and Phase I, Phase II, COX-1, and COX-2, and PGE2 levels in acute and chronic estrogen replacement

| Treatment | Phase I | Phase II | COX-1 | COX-2 | PGE2 |
|----------------|----------------------------------|----------------------------------|---|--|---------------------------------|
| Chronic | | | | | |
| Overall | $R^2=.009$; $m=.04 \pm .153$ | $R^2=.155$; $m=.35 \pm .308$ | $R^2=.115$; $m=-.736^{-4} \pm .526^{-4}$ | $R^2=.072$; $m=-.493^{-4} \pm .456^{-4}$ | $R^2=.513$; $m=.040 \pm .013$ |
| Vehicle | $R^2=.022$; $m=-.041 \pm .155$ | $R^2=.208$; $m=.410 \pm .461$ | $R^2=.205$; $m=-.107^{-3} \pm .794^{-4}$ | $R^2=.156$; $m=-.862^{-4} \pm .757^{-4}$ | $R^2=.843$; $m=.064 \pm .013$ |
| Estradiol | $R^2=.574$; $m=-1.289 \pm .784$ | $R^2=.785$; $m=2.523 \pm .931$ | $R^2=.023$; $m=-.289^{-4} \pm .763^{-4}$ | $R^2=.327^{-3}$; $m=-.242^{-5} \pm .547^{-4}$ | $R^2=.152$; $m=.014 \pm .019$ |
| Acute | | | | | |
| Overall | $R^2=.008$; $m=-.013 \pm .085$ | $R^2=.207$; $m=-.160 \pm .180$ | $R^2=.197$; $m=.352^{-3} \pm .237^{-3}$ | $R^2=.043$; $m=.722^{-4} \pm .112^{-3}$ | $R^2=.459$; $m=.810 \pm .292$ |
| Vehicle | $R^2=.014$; $m=-.022 \pm .093$ | $R^2=.253$; $m=-.508 \pm .435$ | $R^2=.031$; $m=-.143^{-3} \pm .301^{-3}$ | $R^2=.008$; $m=-.323^{-4} \pm .135^{-3}$ | $R^2=.254$; $m=.117 \pm .076$ |
| Estradiol | $R^2=.444$; $m=-.268 \pm .173$ | $R^2=.068$; $m=-.564 \pm 1.200$ | $R^2=.006$; $m=.403^{-4} \pm .206^{-3}$ | $R^2=.078$; $m=-.610^{-4} \pm .855^{-4}$ | $R^2=.193$; $m=-.479 \pm .399$ |

Bold items indicate significant correlations ($P < .05$). m =slope.

5%]×hormone condition [control or estradiol]) to determine significant differences in serum levels of CORT. To determine significant changes in PGE2 serum levels, a three-way ANOVA was used (formalin×hormone condition×length of estradiol treatment [acute or chronic]). Fisher's least significant difference *post hoc* testing was done when appropriate. COX protein levels were expressed as a percentage of their control group. To analyze differences in COX-1 and COX-2 protein levels between estradiol and their respective controls, *t*-tests were done. Within each estradiol replacement paradigm, linear regressions were run comparing CORT serum levels with PGE2 serum levels, COX-1 and COX-2 protein levels, or formalin-induced behavioral responses. For all comparisons, significance was at the .05 level.

RESULTS

In chronic-estradiol-treated rats, estradiol administration significantly increased CORT serum levels ($F(1, 35)=9.63$; $P < .01$; Figure 1A). Furthermore, higher serum levels of CORT were observed in rats administered formalin than in vehicle-treated rats ($F(2, 35)=80.98$; $P < .01$; Figure 1A). COX-2 protein levels were 25% lower in estradiol-vehicle rats than in vehicle-vehicle rats ($t(6)=3.32$; $P < .05$; Fig. 2B). PGE2 serum levels in rats administered formalin (1% or 5%) did not differ significantly from those in vehicle-treated rats ($F(2, 16)=47.38$; $P > .05$; Figure 1C). Although a positive correlation (see Table 1) between serum levels of CORT and PGE2 was found ($P < .05$), a negative correlation between serum levels of PGE2 and COX-1 protein levels was observed ($P < .05$; see Table 2). In vehicle-treated rats, a negative correlation between PGE2-COX-2 and PGE2-COX-1 was obtained (Table 2), but a positive correlation between CORT and PGE2 was

observed. After chronic estradiol administration, a positive correlation of PGE2 and COX-2 was obtained (Table 2).

After acute estradiol administration, serum levels of CORT were significantly higher in formalin-treated rats than in vehicle-treated rats ($F(2, 31)=17.39$; $P < .01$; Figure 1B). Although no changes in COX-1 and COX-2 protein levels were observed (Figure 2 D-E), PGE2 serum levels were higher in rats administered 5% formalin than in vehicle- and 1%-formalin-treated rats ($F(2, 16)=47.38$; $P < .01$; Figure 1C). Similar to chronic estradiol-treated rats, those receiving acute estradiol treatment displayed a positive correlation between serum levels of CORT and PGE2 (see Table 1). In vehicle-treated rats, a positive correlation of PGE2 and COX-1 was observed (Table 2). However, after acute estradiol replacement, a correlation between PGE2 and formalin-induced behavioral responses failed to reach significance (.07 and .06, respectively; see Table 2).

Overall, estradiol replacement significantly increased serum levels of PGE2 as compared with vehicle treatment ($F(1, 16)=46.19$; $P < .01$; Figure 1C). Regardless of the manner of estradiol administration, chronic or acute, estradiol altered the serum levels of PGE2. Namely, rats receiving chronic treatment had significantly lower levels of PGE2 than those receiving acute treatment, regardless of the group treatment conditions ($F(1, 16)=124.17$; $P < .01$).

DISCUSSION

Similar to previous observations, chronic estradiol replacement increased CORT serum levels in vehicle-treated rats.^{12,13} However, after acute estradiol replacement, this induction was not observed, suggesting that the length and time of exposure to estradiol may alter hypothalamic-pituitary-adrenal activity. After formalin administration,

Table 2. Correlation of PGE2 and Phase I, Phase II, COX-1, and COX-2 levels in SILASTIC and injected animals

| Treatment | Phase I | Phase II | COX-1 | COX-2 |
|-----------|--|--|---|---|
| Chronic | | | | |
| Overall | R ² =.079; m=-1.216 ± 1.309 | R ² =.857 ⁻³ ; m=-.845 ± 9.131 | R²=.323; m=-.001 ± .706⁻³ | R ² =.064; m=.001 ± .001 |
| Vehicle | R ² =.525; m=-5.147 ± 2.444 | R ² =.161; m=-17.11 ± 19.51 | R²=.731; m=-.003 ± .693⁻³ | R²=.538; m=-.002 ± .826⁻³ |
| Estradiol | R ² =.031; m=.404 ± 1.130 | R ² =.222; m=5.224 ± 4.885 | R ² =.007; m=-.243 ⁻³ ± .001 | R²=.703; m=.005 ± .001 |
| Acute | | | | |
| Overall | R ² =.224; m=.068 ± .040 | R ² =.147; m=.300 ± .227 | R ² =.099; m=.197 ⁻³ ± .148 ⁻³ | R ² =.033; m=.511 ⁻⁴ ± .687 ⁻⁴ |
| Vehicle | R ² =.045; m=.168 ± .387 | R ² =.009; m=.413 ± 2.109 | R²=.424; m=.002 ± .753⁻³ | R ² =.172; m=.573 ⁻³ ± .397 ⁻³ |
| Estradiol | R ² =.600; m=.106 ± .043 | R ² =.621; m=.566 ± .220 | R ² =.234; m=.198 ⁻³ ± .135 ⁻³ | R ² =.162; m=.716 ⁻⁴ ± .615 ⁻⁴ |

Bold items indicate significant correlations (*P*<.05). m=slope.

regardless of the manner of estradiol replacement, CORT levels increased 4-fold, suggesting the involvement of CORT in inflammatory responses. Indeed, CORT has been shown to exert anti-inflammatory actions, with evidence that inflammation contributes to nociceptive responses in different inflammatory models; inflammatory responses to complete Freund's adjuvant are attenuated with glucocorticoids or dexamethasone.¹⁴ We postulate that because neither estradiol replacement paradigm altered CORT responses to formalin, formalin administration may have caused a ceiling effect on CORT release, thus masking the possible estradiol-mediated responses.

CORT has been shown to be directly regulated by serum levels of PGE2.¹⁵ In our study, both models of estradiol replacement produced a significant correlation of PGE2 and CORT levels. These results suggest a strong interaction between COX activity and CORT, regardless of the length of exposure to estradiol. Interestingly, significant changes in COX-2 protein levels were detected in vehicle-treated rats only after chronic estradiol replacement. Because long-term estradiol replacement is associated with regulatory protein transcription (whereas acute estradiol administration is associated with membrane-mediated actions), alterations in COX-2 protein levels in vehicle-treated rats after chronic estradiol replacement may represent estradiol transcriptional regulation in COX-2 expression. However,

because PGE2 serum levels were increased after acute and chronic estradiol administration, it is feasible that estradiol also directly regulates COX-1 and COX-2 activity. Consistent with previous findings, formalin administration did not alter either COX-1 or COX-2 protein levels.¹² These data further support the idea that COX activity rather than protein level alteration may underlie estradiol's hormonal effects on inflammatory responses to pain.

Estradiol has been shown to directly alter the synthesis of prostaglandins in non-central nervous system tissue; for example, estradiol decreased PGF2 synthesis in bovine endometrium.¹⁰ In both of our hormone-control groups (rats injected with sesame oil or implanted with cholesterol SILASTIC), a significant correlation between COX protein levels and PGE2 was found, suggesting that PGE2 levels are directly regulated by COX levels. After chronic estradiol replacement, a positive correlation between PGE2 levels and COX-2 protein levels, but no correlation with COX-1, was observed. However, after acute estradiol administration, no correlation between PGE2 and either COX-1 or COX-2 was observed. Furthermore, estradiol increased overall PGE2 levels, and a further interaction between estradiol and PGE2 was observed in formalin-treated rats. On the basis of these observations, we postulate that estradiol directly alters the synthesis of PGE2. However, the direction of this regulation is affected by the length and

manner of estradiol replacement. Tege-der et al demonstrated that after 5% formalin administration, PGE2 release coincided with nociceptive behavioral responses, which returned to baseline levels approximately 2 hours after formalin injection.¹⁶ On the basis of that report and our own observations, we postulate that during inflammatory stimuli, PGE2 release is regulated in part by circulating estradiol.

Rats receiving chronic estradiol replacement had significantly lower serum levels of PGE2 than those rats receiving acute estradiol replacement. For example, after 5% formalin, acute estradiol treatment produced a 10-fold higher serum level of PGE2 than did chronic estradiol treatment. That these differences were observed in both estradiol- and vehicle-treated rats suggests experimental manipulation (such as injections 48 hours before formalin versus implantation of capsule 1 week before formalin) may underlie hyper-release of PGE2 to the serum. From these observations, we postulate that the differential behavioral responses to different intensities of inflammatory stimuli (5% vs. 1% formalin) elicited by chronic and acute estradiol replacement may be related in part to this hyper-release of PGE2.

In vitro studies have confirmed that PGE2 increases CORT release by the adrenal gland.¹⁵ For example, in adrenocortical tissues and cells, prostaglandins directly stimulate corticosteroidogenesis.¹⁷ Thus, it has been postulated

that CORT and PGE2 have bidirectional regulatory mechanisms; CORT release is regulated by an inflammatory stimulus, and the subsequent PGE2 release further increases CORT secretion. Our study demonstrated a significant positive correlation between serum PGE2 and CORT levels, lending further support to this theory. We postulate that CORT secretion will act on COX-1 and COX-2 activity to decrease further PGE2 release and therefore will decrease the nociceptive behavioral response to formalin administration. In chronic estradiol-treated rats, our study found a negative correlation between PGE2 levels and COX-1 protein levels. This finding also provides support for the theory. Indeed, we have previously found that flinching during Phase I of the behavioral responses after formalin administration of the response positively correlates with endogenous serum corticosterone levels in female rats (see Kuba et al, this issue).

IMPLICATIONS FOR IMPROVING HEALTH DISPARITIES

The administration of exogenous estradiol may mediate this system by regulating the levels of PGE2 and/or CORT released, thereby mediating the nociceptive response to formalin. Estrogen effects in PGE2 and CORT, in

turn, suggest that during the different reproductive stages females may experience differential activation of inflammatory response.

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