The purpose of this study was to evaluate the effects of stress and estradiol (E2) on pain tolerance. Ovariectomized rats were assigned to treatment groups based on a 2 × 4 factorial design comprising stress (nonstress × stress) and hormone treatment vehicle × E2 [0.25 mg/kg/d] × estrogen receptor alpha (ERα)-selective agonist propyl pyrazole triol (1 mg/kg/d) × estrogen receptor beta (ERβ)-selective agonist diarylpropionitrile (1 mg/kg/d). Stressed animals underwent daily 60-minute immobilization for 22 days. Pain tolerance was assessed with the hot plate test, an acute thermal pain test. In this study, stressed rats showed increased (P < .05) pain tolerance compared with nonstressed rats (25.0 ± 1.92 s vs 20.4 ± 1.02 s, respectively). Increased (P < .05) pain threshold was observed in nonstressed and stressed rats treated with E2 and the ERα agonist compared with vehicle-treated rats. Interestingly, the ERβ agonist only increased (P < .10) pain thresholds in stressed rats. Stressed rats exhibited higher (P < .05) β-endorphin levels compared with nonstressed rats in all hormone-treatment groups. With the exception of stressed rats treated with the ERβ agonist, there was no hormone effect on β-endorphin levels. These studies suggest that E2’s effect on pain thresholds may be mediated via the ERα, while the interaction between chronic stress and ERβ may also enhance pain threshold.

Keywords: Estrogen receptor agonists, hot plate test, pain, restraint stress, stress.

Pain is a complex subjective phenomenon the body uses to signal tissue injury and danger. It can involve both sensory and emotional components that can be influenced by physical and psychological factors. Pain is classified by time course (acute to chronic) and severity (mild to complete agony). As one of the most common reasons for which patients seek medical attention, pain can lead to clinical and psychological changes that increase an individual’s morbidity and mortality if not treated properly. Furthermore, delays and inadequate relief of acute pain can lead to changes in the central nervous system (CNS), contributing to the development of chronic and neuropathic pain.

The body’s response to any actual or potential threat is regulated by the hypothalamic-pituitary-adrenal (HPA) axis through hormonal feedback. The HPA axis involves the release of corticotropin-releasing hormone from the hypothalamus, which regulates the secretion of adrenocorticotropic hormone from the anterior pituitary, which then regulates the secretion of glucocorticoid (cortisol in humans and corticosterone in rodents) from the adrenal glands. Glucocorticoid, in turn, provides negative feedback to regulate central pathways. In the context of pain, glucocorticoid has been shown to reduce pain responses and increase pain threshold through the activation of glucocorticoid receptors and secretion of β-endorphin from the pituitary.

In addition, steroid hormones can play an important role in behavioral and hormonal responses to stress and regulation of pain. Increased pain perception has been correlated with periods of low estradiol (E2) during the menstrual cycle. Abundant evidence from both human and animal studies shows that E2 treatment may elicit antinociceptive effects. Interestingly, E2 works synergistically with corticosterone to alter behaviors including mood, cognition, and pain.

Behavioral, neuroendocrine, and autonomic components are involved in homeostasis. Behavioral tests and serum assays of neuroendocrine markers represent reliable methods to quantify stress and pain in rat models. The hot plate test, an acute thermal pain test, is a method to study responses to a noxious stimulus without causing nerve injury. As a supraspinal thermal pain test, the hot plate test requires integration at the brainstem level of the CNS. Pain and temperature systems involve specific
regions of the CNS including axons of the dorsal root ganglion, dorsal horn, spinal cord, and thalamic nuclei located in the diencephalon just superior to the hypothalamus. First-order neurons from the free nerve endings of the periphery synapse on second-order neurons located in the substantial gelatinosa of the spinal cord. These second-order neurons traverse the midline of the spinal cord and ascend to nuclei located in the thalamus. A number of centers located in the thalamus, brainstem, and cortex are responsible for the emotional component of pain. There are also descending pathways from higher centers that network with ascending pathways to modulate the transmission of pain signals to the cortex. Studies show particularly, the locus coeruleus, HPA axis, and the communication of the aversive emotional effect of pain; afferent pathways that operate and relay signals through the limbic area of the brain are of primary importance in communicating the aversive emotional effect of pain; particularly, the locus coeruleus, HPA axis, and the dorsal/ventral noradrenergic bundles. Studies show the transmission of pain is modulated by β-endorphin, a neurohormone.

The purpose of this study was to determine whether E2, when administered under chronic stress conditions (immobilization for 1 h/d for 22 days), modulates responsiveness to an acute painful stimulus. We hypothesized that chronic immobilization stress and E2 would alter pain tolerance, which in turn is mediated by β-endorphin.

Materials and Methods

- **Subjects.** Ninety-six female Sprague-Dawley rats (200-225 g) were purchased from an approved vendor (Harlan, Indianapolis, Indiana). On arrival, rats were randomly housed in pairs in standard shoebox cages (42 x 20.5 x 20 cm) with hardwood chip bedding (Sani-Chip, Laboratory Grade, Harlan Teklad, Madison, Wisconsin), and the entire box was changed 2 to 3 times a week. Rats were housed on a 12-hour reversed light/dark cycle (lights out at noon) with ad libitum access to food and water. The animal facility was maintained at consistent temperatures (21-24°C) and humidity (40%-70%). All rats were acclimated to the facility for 9 days before experimentation. The rats were handled daily during the acclimation period and throughout the entire experiment to become familiar with the investigators. Daily body weights, as well as food and water consumption, were recorded over the length of the study. Rats were weighed individually and food and water consumption was recorded for each pair. All procedures conducted were approved by the Institutional Animal Care and Use Committee and conducted in full compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

- **Experimental Design.** The experiment was based on a 2 x 4 factorial design comparing the effect(s) of stress (nonstress x daily 60-minute immobilization stress) and hormone treatment (VEH; E2; propyl pyrazole triol [PPT], an estrogen receptor alpha [ERα] agonist; and diarylpropionitrile [DPN], an estrogen receptor beta [ERβ] agonist). Rats were assigned to 1 of the 8 treatment groups by counterbalancing based on body weights taken after acclimation. To accommodate for consistent procedural times and behavioral testing, the study was designed to run 16 or 24 rats at a time, with each run including representatives from all treatment groups. Briefly, after acclimation, the rats were ovariectomized (OVX) and implanted with hormonal treatments delivered by an Alzet osmotic pump (Alzet Model 2002; Durect Co, Cupertino, California). Rats assigned to the stressed groups underwent daily immobilization stress during the experimental phase, which was initiated 3 days after surgery and lasted for a total of 22 days. All rats were tested for pain tolerance using the hot plate test during week 3 of the experimental phase. At the end of treatment, the rats were euthanized by carbon dioxide overdose (via gaseous delivery to the home cage) with rapid decapitation. Trunk blood of each animal was obtained immediately following decapitation into 50-mL conical flasks containing 150 units of heparin, centrifuged (1,000 × g) for 20 minutes. Plasma was harvested and stored at -80°C until further analysis.

- **Drugs.** Estradiol 17-β (Sigma Chemical Co, St Louis, Missouri) binds with nearly equal affinity to both estrogen receptors. To study the respective biological roles of ERα and ERβ, specific estrogen receptor agonists PPT and DPN were also purchased (Tocris Cookson, Inc, Ellisville, Missouri). Hydroxypropyl beta cyclodextrin (27%) (Sigma Chemical) served as VEH.

- **Surgery.** After acclimation, all rats underwent bilateral ovariectomy and Alzet osmotic pump implantation under isoflurane anesthesia. Animals were subcutaneously implanted with the Alzet osmotic pumps between the shoulder. Pumps contained VEH, E2, PPT, or DPN. The E2, PPT, and DPN were dissolved in 27% hydroxypropyl beta cyclodextrin (Sigma Chemical). Because of pump reservoir capacity, new pumps were rapidly replaced (less than 2 minutes after anesthesia) under sterile conditions and isoflurane anesthesia at 13 days after the initial pump insertion. The pump insertion site was monitored daily for any unusual bleeding, drainage, or swelling (no animals displayed these effects at any point during the study). Pumps delivered 0.25 mg/kg body weight (bw) of E2 (subcutaneous [sc]), 1 mg/kg bw of PPT (sc) and 1 mg/kg bw of DPN (sc). This dose represents physiological levels of circulating E2 between the proestrous period. The doses for estrogen receptor agonists PPT and DPN represent previously established doses for these agonists. Control animals in both the stress and nonstress groups received VEH (hydroxypropyl beta cyclodextrin) only.
- **Immobilization Stress.** Animals assigned to stress groups underwent immobilization (3 days after OVX) using finger-like restraining devices (Centrap Cages, Fisher Scientific, St Louis, Missouri) for 60 min/d. Rats were placed in the Centrap cage and the restraining “fingers” were tightened until the animals were immobilized but not compressed, pinched, or screaming/screeching. Animals were unable to turn or barrel-roll in the Centrap cage. Studies show that repeated immobilization stress (most significantly at 21 days) causes structural remodeling in areas of the brain responsible for emotional memories and regulation of the stress response (amygdala, hippocampus, and prefrontal cortex).\(^{25,26}\)

- **Hot Plate Test.** The hot plate test is an acute thermal pain test used to measure the latency to hind paw lick, which is considered indicative of an antinociceptive response. The testing apparatus, consisting of a clear plastic box with a flat metal surface, was heated to 51°C. Each rat was sequentially tested 3 times at 10-minute intervals. All animals were returned to their cages after each test session. Basal latency was determined as the average of the 3 measurements. The exposure to the thermal stimulus lasted no more than 60 seconds. During the baseline phase, animals were acclimated to the hot plate test using the same protocol. Testing occurred in the late part (week 3) of the experimental phase. All nonstressed animals were tested first, followed by stressed animals. Testing started 1 hour after the start of the dark phase, and stressed animals were tested within 20 minutes after removal from restraints. The hot plate platform and box were cleaned with 35% ethanol solution between animals to minimize olfactory cues.

- **Enzyme-Linked Immunoabsorbent Assay (ELISA).** Serum concentrations of beta endorphin were measured using a standard ELISA (Phoenix Pharmaceuticals, Inc, Burlingame, California). Assay plates were read using a VICTOR X5 Multilabel Reader (Perkin Elmer Life and Analytical Sciences, Shelton, Connecticut) and raw data analyzed using manufacturer’s protocol (Phoenix Pharmaceuticals). Statistical analyses were conducted using a 2-way ANOVA with treatment groups of stress or hormone treatment (VEH, E2, PPT, and DPN) as independent variables followed by Tukey’s post hoc test.

- **Data Analysis.** Hot plate results on latency to hind paw lick were expressed as mean latency ± standard error of the mean and converted to percent maximal possible effect (% MPE) according to the formula: (TL-BL)/(60-BL) × 100, where TL = test latency and BL = basal latency. Statistical significance of differences for pain sensitivity was determined by either \( t \) test or 2-way analysis of variance (ANOVA) with a random block design. After an overall significant treatment effect was determined with the ANOVA, a Fishers LSD post hoc test was conducted to determine differences between groups.

**Results**

- **Effects of Stress and Hormone Treatment on Nociception.** Following daily 1-hour immobilization, VEH-treated stressed rats showed increased (\( t \) [21] = −2.09, \( P < .05 \)) latency to hind paw lick during the hot plate test compared to the VEH-treated nonstressed rats (25.0 ± 1.9 s vs 20.4 ± 1.0 s, respectively) to suggest a decreased sensitivity to pain (Figure 1). When comparing the effect of stress and hormone treatment, a significant main effect of hormone treatment (\( F \) [1, 84] = 7.85, \( P < .05 \)) and of stress (\( F \) [1, 84] = 6.47, \( P < .05 \)) was observed. Both nonstressed and stressed groups treated with E2 and PPT (ER\( \alpha \) agonist) showed an increase (\( P < .05 \)) in latency to hind paw lick compared with their respective controls, whereas there was no ER\( \beta \) agonist effect (\( P > .10 \)) in the nonstressed group (see Figure 2).

- **Effects of Stress and Hormone Treatment on Plasma \( \beta \)-endorphin Levels.** Serum level of \( \beta \)-endorphin was analyzed using an ELISA. There was a significant main effect of stress (\( F \) [1, 77] = 02.18, \( P < .05 \)), treatment (\( F \) [3, 77] = 17.72, \( P < .05 \)), and stress treatment interaction (\( F \) [3, 77] = 14.95, \( P < .05 \)). Stressed rats showed an increase (\( P < .05 \)) in \( \beta \)-endorphin levels compared to nonstressed rats in all hormone treatment groups (Figure 3). In nonstressed animals, \( \beta \)-endorphin levels were not altered by hormone treatments (\( P > .10 \)). Among the stressed rats, E2 and PPT treatment did not affect (\( P > .10 \)) circulating \( \beta \)-endorphin levels compared with VEH treatment, whereas DPN (ER\( \beta \))-treated rats had higher (\( P < .05 \)) circulating \( \beta \)-endorphin levels compared with all other groups.

**Discussion**

In the present study, chronic daily immobilization stress enhanced pain tolerance in OVX rats by increasing the latency to hind paw lick during hot plate testing. Furthermore, E2 and PPT, the ER\( \alpha \) selective agonist, enhanced pain tolerance compared with controls in both the nonstressed and stressed animals. Interestingly, treatment with the ER\( \beta \) selective agonist DPN appears to enhance tolerance to pain only when a rat is chronically stressed. Lastly, stressed rats have increased peripheral levels of \( \beta \)-endorphin to suggest its involvement in the increase in pain threshold during acute thermal pain.

Pain threshold is modulated by both acute and chronic stress. Acute stress has been shown to have antinociceptive effects immediately following the stressor.\(^{27}\) More controversial are the nociceptive effects experienced during chronic stress conditions, which show mixed antinociceptive effects.\(^{27-29}\) Additionally, the interactive effect of chronic stress and E2 on response to pain remains unknown. The predominant use of male animals
as subjects and acute stress as the paradigm of choice in the current literature explains the inadequacies. For example, chronic immobilization in male rats is hyperalgesic when tested by the temporomandibular joint (TMJ) formalin test or tail-flick response. In this study our results showed that chronically stressed OVX rats showed an increase in pain threshold when exposed to an acute thermal pain assay. These findings suggest that chronic stress may alter pain perception in female rats.

Our results are consistent with other reports showing that the administration of E2 enhanced pain tolerance in both nonstressed and stressed animals. In the present study, under the conditions of stress, E2 enhancement of pain tolerance appears to be mediated by ERα. Tsao and coworkers showed that OVX rats receiving E2 treatment had decreased autonomy (self-mutilation) scores after sciatic nerve ligation compared to rats not treated with E2. Moreover, OVX rats treated with E2 had decreased TMJ nociception. This effect is possibly mediated independent of opioid receptors through a nitric oxide/cyclic guanosine monophosphate signaling pathway. ERα is expressed and activated by pain in the medullary dorsal horn, a region critical in nociceptive transmission, providing support for our finding that ERα stimulation may directly regulate pain transmission at specific levels of the spinal cord. Interestingly, there does not appear to be an additive effect of the ERα agonist and stress on pain tolerance. It is possible that the enhancement of pain tolerance may be a convergence of effects downstream from the stress and activation of ERα.

In contrast, our study showed that treatment with the ERβ-selective agonist DPN appears to enhance tolerance to pain only when a rat is chronically stressed. Previous studies have shown that ERβ messenger RNA in the paraventricular nucleus of the hypothalamus is up-regulated by stress and is corticosterone-dependent. Although it is unclear how this can occur because proopiomela-
necortin (POMC) neurons are not known to colocalize with ERβ-positive neurons, our data suggest chronically stressed animals become more sensitive to DPN administration by enhancing the effect of stress on pain perception, and may do so hormonally via the secretion of β-endorphin.

In the present study, reduced pain sensitivity in the animals that were stressed by immobilization is consistent with clinical observations. For example, combat veterans with posttraumatic stress disorder (PTSD) reported reduced pain sensitivity during fixed-temperature (43°C) thermal pain testing compared with those who do not have PTSD. Similar responses exhibited by animals exposed to repeated immobilization in the manner described in this study include exaggerated startle and depressive behaviors. Collectively, our studies along with others strongly suggest that the rodent immobilization model simulates the human perception of a lack of control associated with greater severity of symptoms after a traumatic experience. Although immobilization stress appears to be a mild stressor, it has been found to induce fear conditioning when applied to rats and potentiate anxiety. Inflammatory mediators are released in response to stressful stimuli. In turn, the CNS signals the activation of the HPA axis and the sympathetic-adrenal axis. Stress produces an increase in the adrenergic activity of the hypothalamus. In response to the increase in adrenergic activity, POMC is released and cleaved to produce β-endorphin.

In conclusion, under the conditions of the present study chronic immobilization stress decreases pain sensitivity to acute thermal pain and increases peripheral levels of β-endorphin. This finding suggests stress-induced analgesia may be a result of μ-opioid receptor stimulation. Furthermore, E2 administration-enhanced pain tolerance during hot plate testing may be mediated via ERα. ERβ activation reduced pain sensitivity only in stressed rats; in part, this activation could be acting through the up-regulation of β-endorphin to depress pain afferents. Additional studies are needed to determine interactions with neuromediators or neurotransmitters such as dopamine, histamine, serotonin, and/or excitatory amino acids, which can alter nociceptive responses. The role of E2 administration in regulating the expression of these receptors in response to pain and/or stress will be of importance in determining the underlying mechanisms of action.

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