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Estrogen alters baseline and inflammatory-induced cytokine levels independent from hypothalamic–pituitary–adrenal axis activity

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ABSTRACT

Although estrogen reduces inflammatory-mediated pain responses, the mechanisms behind its effects are unclear. This study investigated if estrogen modulates inflammatory signaling by reducing baseline or inflammation-induced cytokine levels in the injury-site, serum, dorsal root ganglia (DRG) and/or spinal cord. We further tested whether estrogen effects on cytokine levels are in part mediated through hypothalamic–pituitary–adrenal (HPA) axis activation. Lumbar DRG, spinal cord, serum, and hind paw tissue were analyzed for cytokine levels in 17β-estradiol-(20%) or vehicle-(100% cholesterol) treated female rats following ovariectomy/sham adrenalectomy (OVX), adrenalectomy/sham ovariectomy (ADX) or ADX + OVX operation at baseline and post formalin injection. Formalin significantly increased pro-inflammatory interleukin (IL)-6 levels in the paw, as well as pro- and anti-inflammatory cytokine levels in the DRG, spinal cord and serum in comparison to naïve conditions. Estrogen replacement significantly increased anti-inflammatory IL-10 levels in the DRG. Centrally, estradiol significantly decreased pro-inflammatory tumor necrosis factor (TNF)-α and IL-1β levels, as well as IL-10 levels, in the spinal cord in comparison to cholesterol treatment. At both sites, most estradiol modulatory effects occurred irrespective of pain or surgical condition. Estradiol alone had no influence on cytokine release in the paw or serum, indicating that estrogen effects were site-specific. Although cytokine levels were altered between surgical conditions at baseline and following formalin administration, ADX operation did not significantly reverse estradiol's modulation of cytokine levels. These results suggest that estrogen directly regulates cytokines independent of HPA axis activity *in vivo*, in part by reducing cytokine levels in the spinal cord.

1. Introduction

Estrogen deficiency renders postmenopausal women vulnerable to degenerative conditions such as osteoarthritis, osteoporosis, atherosclerosis, and Alzheimer's disease [1–4]. An issue neglected by the current literature is how estrogen alters inflammation-mediated pain responses and reduces neuroinflammatory diseases. This topic is of clinical relevance because elucidating the mechanisms of estrogen's effects on inflammatory responses will greatly enhance our understanding of painful pathological conditions in women and help to understand why postmenopausal women are more susceptible to these severe chronic conditions.

It has been argued that neuronal inflammation is a pro-inflammatory cytokine-mediated process that results from systemic or direct neuronal tissue injury [5]. During inflammation, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 are released by a variety of cells and excite nociceptors either by direct action or indirectly by stimulating the release of inflammatory mediators such as bradykinin, prostaglandin (PG) and substance P [6]. While administration of pro-inflammatory cytokines to rodents produces persistent pain and hyperalgesia, knockout mouse models or antagonists of these cytokines reduce hyperalgesia in animal models of inflammatory and neuropathic pain [7–13]. In the spinal cord, mRNA expression and protein levels of pro-inflammatory cytokines have been identified in response to peripheral or spinal nerve injury [14–17]. On

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the other hand, the anti-inflammatory cytokine IL-10 produces hypoalgesic responses to inflammatory pain; repeated intrathecal injections of plasmid DNA encoding IL-10 reverses allodynia induced by neuropathic pain [18]. IL-10's analgesic effect has been postulated to be via inhibition of TNF-α, IL-1β, and PGs in the dorsal root ganglia (DRG) and spinal cord [19,20].

The sex hormone estrogen has been shown to display anti-inflammatory and antinociceptive properties during inflammation; for instance, in the formalin assay for inflammatory pain, estrogen administration (via SILASTIC capsule) significantly reduces Phase II nociceptive flinching responses in ovariectomized rats and does so dose-dependently [21–23]. Estradiol administration lowers thermal, mechanical and adjuvant-induced hyperalgesia in rats [24,25]. Furthermore, estradiol reduces carrageenan-induced pleurisy and acute inflammation [26,27]. Parts of the anti-inflammatory effects of estrogen have been linked to its interactions with adrenergic and serotonergic systems [25]. However, the full mechanisms underlying estrogen's anti-hyperalgesic effects are still under investigation.

Estrogen has been shown to regulate cytokine activity, thereby offering another mechanism for estrogen-mediated analgesia. Estrogen prevents lipopolysaccharide (LPS)-induced microglial toxicity by attenuating the release of TNF-α and IL-1β [28]. Estrogen replacement lowers IL-6 production in ovariectomized mice, while activation of estrogen receptors (ER) ERα and ERβ leads to IL-6 gene suppression [29,30]. Following collagen-induced arthritis, ethinyl estradiol lowers T cell IL-6 and TNF-α secretion and decreases cytokine and chemokine mRNA levels in joint tissue of mice [31]. Pretreatment of 17β-estradiol to rat primary astrocyte cultures attenuates LPS-induced TNF-α and IL-1β release [32]. Moreover, 17β-estradiol stimulates early cytokine release following spinal cord injury in rats [33]. Because of the known involvement of estrogen in cytokine release and production, it is feasible that the attenuation of nociceptive responses after estradiol administration is in part mediated by impeding cytokine activity. Indeed, blocking TNF-α with infliximab reduces ovariectomy-induced mechanical and thermal hyperalgesia in rats, indirectly suggesting that TNF-α plays an important role in estrogen deficient hyperalgesia [34].

Glucocorticoid (GC) hormones released from the hypothalamic–pituitary–adrenal (HPA) axis also contribute to the control of nociception and inflammation by reducing pro-inflammatory cytokine levels [35–37]. Elevated plasma GC levels suppress IL-1 and TNF-α, and increase anti-inflammatory cytokine IL-10 and IL-4 production [35,36]. Chensue et al. [38] found that during LPS administration, decreased TNF-α levels correlate with elevated release of the GC corticosterone (CORT). Kapcala et al. [39] showed that adrenalectomized rats have increased mortality rates following IL-1β administration, but CORT replacement attenuates that lethality. Injection of metyrapone plus aminoglutethimide (GC inhibitors) increases basal hypothalamic IL-1β mRNA expression and alters IL-1β levels following acute stress in rats [40]. Moreover, estrogen has been shown to modulate GC expression; for instance, estradiol increases CORT levels and following adrenalectomy + ovariectomy (ADX + OVX) estradiol leads to increased GC levels in rodent brain and spinal cord tissue [41–44]. Regulation of hormones released from the HPA axis may be another possible mechanism by which estrogen attenuates cytokine activity during inflammation and tissue injury.

We have previously shown that estrogen replacement reduces formalin-induced flinching responses following OVX and/or ADX in female rats [21]. In this paradigm, however, it is not understood if estrogen modulates inflammatory signaling by regulating cytokine activity in combination with or independent from its regulation of the HPA axis. Furthermore, it is not known whether estrogen modulates basal inflammatory mediators before the introduction of inflammation and/or injury. In this study we postulate that estrogen alters inflammatory mediators by reducing the release of pro-inflammatory cytokines and increasing anti-inflammatory IL-10 levels at the site of injury, systemically, as well as in the central nervous system before and following formalin administration. We further investigate whether hormonal influences from the HPA axis mediate estrogen's effects on regulating cytokine levels.

2. Materials and Methods

2.1. Animals

Eight-week old OVX/sham ADX (denoted as OVX), ADX/sham OVX (denoted as ADX), and ADX + OVX female Sprague-Dawley rats (n = 7–10 per group) were purchased from Taconic (Germantown, NY). Rats were double-housed on a 12-hour light/dark photoperiod (lights on 8 AM EST) with food and water available *ad libitum*. ADX rats were maintained on water supplemented with 0.9% sodium chloride. Rats weighed about 220 g at the time of sacrifice. Animal treatment was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD) and was approved by the Institutional Animal Care and Use Committee at Hunter College of The City University of New York.

2.2. Estradiol replacement paradigm and formalin administration

Two weeks after OVX and/or ADX, a SILASTIC capsule (1 cm, 0.058 in. ID × 0.077 in. OD, Dow Corning) was inserted into the nape of the rats' neck. The capsule contained either 20% 17β-Estradiol (1, 3, 5 [10]-Estratriene-3, 17 Beta-diol; Sigma-Aldrich) in cholesterol (5-Cholestin-3Beta-ol; Sigma-Aldrich, St. Louis, MO) for the experimental group or 100% cholesterol for the vehicle group. This dose and manner of estradiol replacement was chosen because (1) serum estradiol levels were maintained at a steady state [23], (2) it produces physiological conditions similar to rats in proestrus [23], and (3) it reduces formalin-induced behavioral responses [21–23,45].

Using a 27½-gauge needle, rats received a 50 μL formalin injection [5% formaldehyde (Sigma); 95% sterile isotonic saline (0.9% Sodium Irrigation USP, Braun Medical, Irving, CA)] subcutaneously into the intraplantar region of the right hind paw while they were manually restrained. Formalin was administered between 9:00 AM and 3:00 PM. This formalin dose and manner of administration has been shown to produce persistent nociception in OVX female rats [21–23,45]. In order to mimic the time that behavior during the formalin assay is collected, OVX, ADX and ADX + OVX rats were sacrificed by rapid decapitation 60 minutes post formalin injection following a brief (20 sec) exposure to CO_2. In a separate set of animals, naïve OVX, ADX and ADX + OVX rats were administered either 17β-estradiol or cholesterol and were sacrificed two weeks later to establish baseline hormonal replacement effects. For all procedures, rats were randomly assigned to their respective groups.

2.3. Sample collection and cytokine multiplex assay

Upon decapitation, trunk blood was collected in tubes containing K_2-EDTA (BD Vacutainer Systems, Franklin Lakes, NJ), centrifuged at 2600 RPM for 30 min at 4 °C, and serum was stored at −80 °C. Hind paw measurements were taken using a dial caliper (General Tools, New York, NY) measuring the width and depth (the measurement of the plantar to the center dorsal surface) in millimeters (mm). Paws were removed post mortem, weighed