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PERIPHERAL ESTRADIOL INDUCES TEMPOROMANDIBULAR JOINT ANTINOCICEPTION IN RATS BY ACTIVATING THE NITRIC OXIDE/CYCLIC GUANOSINE MONOPHOSPHATE SIGNALING PATHWAY

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Abstract—Recently, we have reported that high physiological estradiol level during the proestrus phase of the estrous cycle or systemic estradiol administration in ovariectomized rats decreases formalin-induced temporomandibular joint nociception. However, the mechanisms underlying the antinociceptive effect of estradiol are presently unknown. In this study, we used the temporomandibular joint formalin model in rats to investigate whether estradiol decreases nociception by a peripheral non-genomic mechanism, and if so, whether this mechanism is mediated by the activation of the nitric oxide–cyclic guanosine monophosphate signaling pathway and of opioid receptors. The administration of estradiol into the ipsilateral, but not into the contralateral temporomandibular joint significantly reduced formalin-induced temporomandibular joint nociception in ovariectomized and diestrus but not in proestrus females. However, the administration of the estrogen receptor antagonist ICI 182780 into the ipsilateral, but not into the contralateral temporomandibular joint blocked the antinociceptive effect of serum estradiol in proestrus females, suggesting that the physiological effect of estradiol in nociception is mediated, at least in part, by a peripheral mechanism. The administration of estradiol into the ipisilateral temporomandibular joint did not affect formalin-induced nociception in male rats. The antinociceptive effect of temporomandibular joint estradiol administration in ovariectomized and diestrus females was mimicked by estradiol conjugated with bovine serum albumin, which does not diffuse through the plasma membrane, and was blocked by the estrogen receptor antagonist ICI 182780. The administration of the nitric oxide synthase inhibitor (nitro-L-arginine) or of a guanylate cyclase inhibitor (1H-(1,2,4)-oxadiasolo (4,2-a) quinoxalin-1-one) into the ipsilateral, but not into the contralateral temporomandibular joint blocked the antinociceptive effect of estradiol and of estradiol conjugated with bovine serum albumin, while the opioid receptor antagonist naloxone had no effect. These findings suggest that estradiol decreases temporomandibular joint nociception in female rats through a peripheral non-genomic activation of the nitric oxide–cyclic guanosine monophosphate signaling pathway.

Key words: estradiol, nociception, temporomandibular joint pain, nitric oxide, cGMP, formalin.

The majority of chronic pain conditions (Unruh, 1996) such as temporomandibular dysfunctions (TMDs) (Dworkin et al., 1990) are more prevalent and severe in women than in men. These findings may apparently suggest a pronociceptive effect of estradiol, as supported by some animal studies (Bereiter, 2001; Cairns et al., 2002; Okamoto et al., 2003). However, our findings that formalin or glutamate-induced temporomandibular joint (TMJ) nociception in rats (Fischer et al., 2008) and those that TMD pain in women (LeResche et al., 2003) is higher during low estradiol times of the reproductive cycle suggest that estradiol attenuates TMJ pain. This suggestion is also supported by other studies in humans (Smith et al., 2006) and in animals (Gaumond et al., 2002; Ceccarelli et al., 2003; Pajot et al., 2003; Kuba et al., 2006; Mannino et al., 2007) that use other pain models. Based on our previous animal studies, we suggest that the lower prevalence of TMJ pain in men may result from a protective effect of testosterone that reduces the risk of males developing TMJ pain (Fischer et al., 2007) and the higher severity of TMJ pain in women may result from the estrogen fluctuation during reproductive cycle, in that TMJ pain is increased during low serum estradiol levels (Fischer et al., 2008).

Although the mechanisms underlying the antinociceptive effect of estradiol are presently unknown, estradiol might induce antinociception, at least in part, by a peripheral non-genomic mechanism. This idea is supported by "*in vitro*" studies showing that estradiol inhibits calcium channel currents in neurons of the dorsal root ganglia via activation of membrane estrogen receptors (Lee et al., 2002; Chaban et al., 2003). In contrast to estrogen receptors located in the cytosol, those located on the membrane mediate the rapid non-genomic effects of estradiol through the activation of second messenger cascades (Evrard and Balthazart, 2004), including the L-arginine–nitric oxide (NO)–cyclic guanosine mono-phosphate (cGMP) pathway (L-arg–NO–cGMP), as demonstrated in endothelial cells (Stefano et al., 2000). This pathway involves the synthesis of NO from L-arginine and the subsequent activation of the guanylate cyclase, leading to increased levels of cGMP, a second messenger that has been associated with peripheral antinociception, including that mediated by peripheral opioids (Durate et al., 1990; Pol, 2007). Estradiol via activation of non-genomic mechanisms might induce antinociception by increasing the release of endogenous opioids

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Abbreviations: cGMP, cyclic guanosine mono-phosphate; DMSO, dimethyl sulfoxide; E–BSA, estradiol coupled to bovine serum albumin; L-NNA, nitro-L-arginine; NO, nitric oxide; ODQ, 1H-(1,2,4)-oxadiasolo (4,2-a) quinoxalin-1-one; OVX, ovariectomized; TMJ, temporomandibular joint.

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by opioid peptide-containing immune cells (Rittner et al., 2008) and/or by interacting with opioid receptor-coupled second messenger systems (Lagrange et al., 1995; Brown et al., 2007). In fact, peripheral opioid mechanisms mediate the antinociceptive effect induced by pregnancy hormones in the rat's TMJ (Arthuri et al., 2005). Therefore, in this study we have used the TMJ formalin model to investigate whether estradiol decreases nociception by a peripheral non-genomic mechanism, and if so, whether this mechanism is mediated by the activation of the NO–cGMP signaling pathway and of opioid receptors.

EXPERIMENTAL PROCEDURES

Animals

This study was carried out in 200–300 g ovariectomized (OVX, $n=223$), diestrus ($n=128$) and proestrus ($n=43$) female and in male ($n=41$) Wistar rats. All efforts were made to minimize the number of animals used and their suffering. The animals were used in experiments when they were 60–75 days old; the mean weights of OVX females (266.2±7.8) were significantly greater (*t*-test, $P=<0.05$) than that of intact proestrus and diestrus females (225.0±5.8).

To investigate a peripheral estradiol-mediated effect we firstly used OVX females because the depletion of serum estradiol level possibly facilitates its assessment. Then we included diestrus and proestrus females because they represent a physiological condition of low and high serum estradiol level, respectively. Male rats were also included because the antinociceptive effect of systemic estradiol is sex specific (Fischer et al., 2007; 2008) and therefore we asked if the peripheral effect might also be.

All animal experimental procedures and protocols were approved by the Committee on Animal Research of the University of Campinas and are in accordance with IASP guidelines for the study of pain in animals (Zimmermann, 1983). The animals were maintained on a temperature-controlled room (±23 °C) and were housed in plastic cages (45×30×15 cm^3) with soft bedding (five/cage) on a 12:12 light cycle (lights on at 6:00 AM) with food and water available *ad libitum*.

Drugs

Formalin was prepared from commercially available stock formalin (an aqueous solution of 37% of formaldehyde) further diluted in 0.9% NaCl to a concentration of 1.5% (Roveroni et al., 2001); estradiol (17β-estradiol, 0.4, 1.2 μg; (Ceccarelli et al., 2004) and 3.6 μg) was dissolved in propylene glycol; estradiol coupled to bovine serum albumin (E–BSA, 1.2 μg of estradiol plus BSA); the NO synthase inhibitor nitro-L-arginine (L-NNA 22 μg; (Toda et al., 1993)) and the opioid receptor antagonist naloxone (10 μg; (Eisenberg et al., 1996), and 30 μg) were dissolved in 0.9% NaCl. The selective estrogen receptor antagonist ICI 182780 (0.16, 1 and 6 μg; (Ceccarelli et al., 2004) and the guanylate cyclase inhibitor 1H-(1,2,4)-oxadiasolo (4,2-a) quinoxalin-1-one (ODQ 0.8 and 8 μg; (Cunha et al., 1999) were dissolved in dimethyl sulfoxide (DMSO)). Formalin, estradiol, E–BSA, naloxone and L-NNA were purchased from Sigma Aldrich, St. Louis, MO, USA; ODQ and ICI182780 were purchased from Tocris Bioscience, St. Louis, MO, USA.

Steroid hormones conjugated with bovine serum albumin have been extensively used to assess their non-genomic effects (Kelly and Levin, 2001). However, it was suggested that E–BSA has biological activity not observed with estradiol. For this reason, the experiments were performed using both 17β-estradiol and E–BSA. The dose–response curves of all drugs were performed in OVX females and the most effective dose was selected for further experiments.

Estrous phase determination

Estrous phase was determined by daily microscope examination of vaginal smears between 7 and 8 AM. In the day of the experiment, estrous phase was confirmed before and immediately after each experiment to ensure that the rats remained in the same phase. Proestrus phase and the initial phase of diestrus (first 4 h) were identified by the predominance (>70%) of nucleated epithelial cells and leukocytes, respectively (Butcher et al., 1974) in rats with at least two consecutive regular 4–5 day cycles. These phases were chosen because they represent phases of high and low ovarian hormonal level, respectively (Butcher et al., 1974).

Gonadectomy

Ovariectomy (45 days old females; (Gordon and Soliman, 1994)) was performed through bilateral upper flank incisions. The ovarian bundles were tied off with 4-O silk sutures and the ovaries removed. The fascia and the skin were closed with 4-O silk sutures. Rats of two experimental groups were sham operated and underwent a surgical procedure similar to that of OVX animals, except that the ovaries were not removed. These groups were used to demonstrate that the surgical procedure does not affect the nociceptive behavior response in diestrus and proestrus females (naïve vs. sham). The procedures were carried out under anesthesia induced by an i.m. injection of a mixture of ketamine (55 mg/kg) and xylazine (5.5 mg/kg). An s.c. injection of ketoprofen (5 mg/kg) was used for post-operative analgesia (Roughan and Flecknell, 2000). OVX and sham-operated rats were used in experiments when they were 3 months of age. The efficacy of ovariectomy was confirmed by the absence of estrous cycle determined by observation of vaginal smears during 10 days.

TMJ injections

The animals were briefly anesthetized by inhalation of halothane to allow the TMJ injection; each animal regained consciousness approximately 30 s after discontinuing the anesthetic. The TMJ injection was performed with a 30-gauge needle introduced into the TMJ at the moment of injection. The contralateral TMJ injections, when necessary, were performed immediately after the ipsilateral injections. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 μl) (Roveroni et al., 2001). The total volume injection varied between 30 and 45 μl, when two or three drugs were injected in the same TMJ, they were administered at a volume of 15 μl per drug in a unique injection. At the conclusion of the behavior test, each animal was anesthetized by an i.p. injection of a mixture of urethane (1 g/kg) and α-chloralose (50 mg/kg). To confirm the correct site of injection, the Evans Blue dye (5 mg/kg) was systemically injected and 15 min later the animals were submitted to cardiac perfusion with normal saline. Since this dye binds to plasma protein, the correct site of injection was indicated by the observation that the plasma extravasation induced by the TMJ injection of formalin was restricted to the TMJ region (Haas et al., 1992).

Testing procedure for TMJ pain

Behavior test was performed during light phase (between 9:00 AM and 5:00 PM) in a quiet room maintained at ±23 °C (Rosland, 1991). Rats did not have access to food or water during the test and each animal was used once. The nociceptive response was assessed by an observer blinded to the experimental manipulation. Before the experiments, each animal was manipulated for 7 days in the test room (handled for approximately 1 min) to be habituated to the experimental manipulation. On the day of the