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ORIGINAL ARTICLE

The antinociceptive effects of estradiol on adjuvant-induced hyperalgesia in rats involve activation of adrenergic and serotonergic systems

Kentaro Okuda · Hideo Iwasaka · Satoshi Hagiwara · Naozumi Takeshima · Junji Takatani · Tetsuya Uchino · Takayuki Noguchi

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Abstract
Purpose Estradiol is a female hormone required for maintaining pregnancy and developing follicles in the ovary. Estradiol has been shown to perform a variety of physiological activities, including pain reduction. In this study, we tested the hypothesis that estradiol exerts antinociceptive effects in a rat model of inflammatory hyperalgesia.
Methods We established a subacute hyperalgesia model using plantar injection of Freund's complete adjuvant (FCA) in Sprague–Dawley rats. We administered estradiol every 24 h, beginning 12 h after FCA administration, and used the plantar test to determine its effect on hyperalgesia. To determine the mechanism of action of estradiol, we evaluated the role of the opioid antinociceptive system using naloxone and the role of the descending pain inhibitory system using the α-2-receptor antagonist yohimbine and the serotonin receptor antagonist methysergide.
Results Administration of FCA induced hyperalgesia, which was significantly reduced by estradiol treatment compared to controls. Moreover, this effect was not antagonized by naloxone, but was attenuated by α-2-receptor and serotonin-receptor antagonists.
Conclusion Estradiol is known to perform a variety of physiological functions. Our findings suggest that one such function is antinociception via an interaction with α-2 receptors and serotonin receptors.

K. Okuda (✉) · H. Iwasaka · S. Hagiwara · N. Takeshima · J. Takatani · T. Uchino · T. Noguchi
Department of Anesthesiology and Intensive Care Medicine, Oita University Faculty of Medicine,
1-1 Idaigaoka-Hasamamachi, Yufu, Oita 879-5593, Japan
e-mail: kent024@oita-u.ac.jp

Keywords Estradiol · Freund's complete adjuvant (FCA) · Hyperalgesia · Naloxone · Descending pain inhibitory system

Introduction

Nerve damage often results in prolonged pain that can continue for a relatively long period of time. This kind of chronic pain is often intractable and difficult to treat. In fact, an estimated 12–80% of people experience chronic pain and 18–63% experience severe disabling pain. Approximately 30% can be treated effectively, whereas 70% show no improvement in pain levels despite treatment [1, 2].

Estradiol, a sex hormone produced primarily in the ovaries, shows a characteristic pattern of secretion that accompanies follicular growth. In addition, a large amount is secreted as part of placental estrogen during pregnancy. Estradiol is also involved in gonadal development and proliferation, bone metabolism, and bone growth [3]. It has been used as a therapeutic agent for ovarian and uterine hypoplasia, menopausal symptoms, irregular menstruation, hypermenorrhea, amenorrhea, menstrual pain, functional infertility, prevention and treatment of osteoporosis, and senile vaginitis [3].

In recent years, the relationship between sex hormones and pain has received considerable attention. For example, a direct relationship between pain and sex hormones is thought to play a role in sex differences in pain perception [4]. Estradiol has been shown to reduce acute pain in rats [5]. Similarly, administering estradiol reduced pain in clinical trials [6]. However, few studies have addressed the mechanism behind these antinociceptive effects.

Pain nerve endings detect physical or chemical stimuli, which are then signaled via afferent nerves in the lateral spinothalamic tract to the posterior central gyrus of the

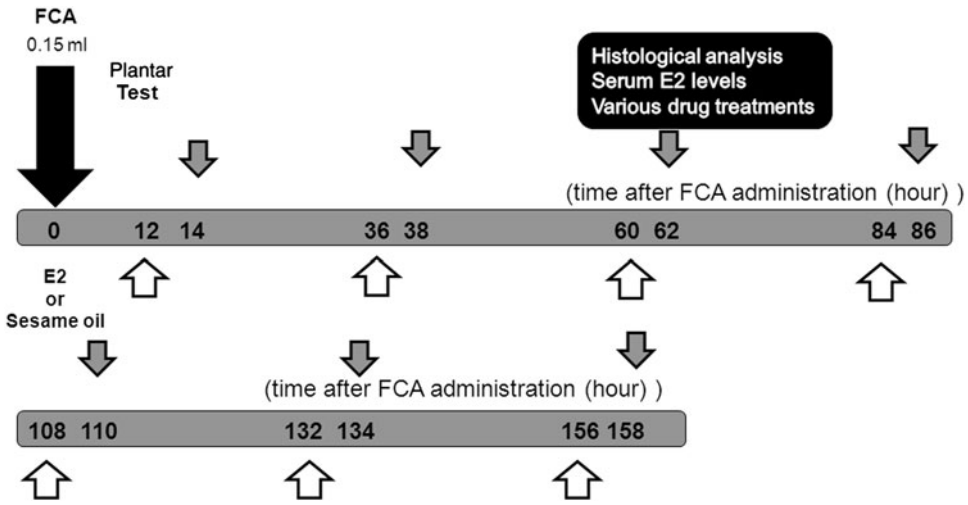

Fig. 1 Timeline of experimental design. *Black arrows*, times of Freund's complete adjuvant (*FCA*) administration; *white arrows*, times of 17β-estradiol (E_2) or *sesame oil* administration; *gray arrows*, times of plantar tests

cerebrum, where they are recognized as pain [7, 8]. Several endogenous mechanisms inhibit pain, including the descending pain inhibitory system that inhibits nociceptive message inputs from peripheral tissue to the spinal dorsal horn [9–11]. In this study, we tested the hypothesis that estradiol administered after the onset of hyperalgesia can act as an antinociceptive agent. We used a Freund's complete adjuvant (FCA)-induced inflammatory hyperalgesia rat model to determine if the descending pain inhibitory system was involved in estradiol effects.

Materials and methods

Animals

Experiments were performed on male Sprague–Dawley rats (250–300 g; Japan Charles River, Yokohama, Japan), individually housed in cages lined with sawdust. Animals were provided with standard laboratory rodent food and water ad libitum. Room temperature was maintained at 22° ± 0.5°C and relative humidity at 60%, under 12-h light and dark cycles (lights on from 0800 to 2000). All testing was conducted during the light cycle. The study was approved by the Ethical Committee of Animal Research at the College of Medicine, Oita University, Oita, Japan. All experiments were conducted in accordance with the Institutional Committee for the Care and Use of Animals and guidelines from the International Association for the Study of Pain on ethical standards for investigations on experimental pain in animals [12].

Drugs

Freund's complete adjuvant was purchased from Pierce (Rockford, IL, USA). Yohimbine and methysergide were purchased from Sigma Chemical (St. Louis, MO, USA).

Induction of adjuvant-induced peripheral hyperalgesia

Baseline withdrawal latency was determined for all animals before the induction of peripheral hyperalgesia. Rats were then sedated by sevoflurane anesthesia (Maruishi, Osaka, Japan) and unilateral hind paw hyperalgesia was induced by injecting 0.15 ml FCA into the right hind paw of each animal. As a control, 0.15 ml saline was injected into each animal's left hind paw. Withdrawal latencies were measured daily for 6 days following FCA administration (Fig. 1).

17β-estradiol treatment

Twelve hours after induction of FCA-induced peripheral hyperalgesia, animals were randomly allocated into three groups according to the following treatments: sesame oil group [sesame oil vehicle, 1 ml/kg, by subcutaneous (s.c.) injection], 1.0 mg/kg E_2 group (17β-estradiol; Sigma-Aldrich, in sesame oil, 1 ml/kg, s.c.), and 0.1 mg/kg E_2 group (17β-estradiol in sesame oil, 1 ml/kg, s.c.). Vehicle or E_2 was administered every 24 h starting 12 h after FCA administration (Fig. 1).

Naloxone treatment

Sixty-two hours after FCA, six rats from each experimental group were given intraperitoneal injections of naloxone (0.5 mg/kg).

Histological analysis

Sixty-two hours after starting the examination, rats were anesthetized with sevoflurane and transcardially perfused with 60 ml warm physiological saline, followed by 300 ml 10% (w/v) formaldehyde. The hind paws were removed, immediately immersed in 10% buffered formalin, embedded

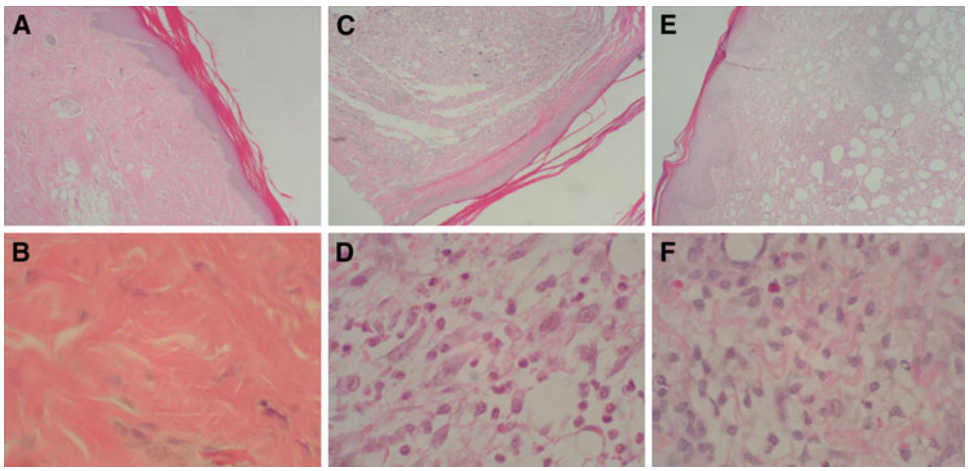

Fig. 2 Histological appearance of subcutaneous tissue, 62 h after injection of FCA. The sesame oil group with saline (**a**, **b**) showed little accumulation of inflammatory cells; however, the same level of inflammatory cell accumulation was observed in the sesame oil group with FCA (**c**, **d**) as in the 1 mg/kg E_2 group with FCA (**e**, **f**) ($n = 6$, each group). Sections were stained with hematoxylin and eosin (H&E) (**a**, **c**, **e** ×40; **b**, **d**, **f** ×400)

in paraffin, and cut into 4-μm-thick sections. Paraffin sections were stained with hematoxylin and eosin (H&E).

Measurement of withdrawal latency by plantar test

Sixty-two hours after starting the examination, withdrawal latency was determined using the plantar test, based on a method modified from Hargreaves et al. [13]. Rats were placed in an acrylic box with a glass pane floor, and the plantar surface of their right hind paw was exposed to a beam of infrared radiation (Ugo Basile; Stoelting, Chicago, IL, USA). Latency of paw withdrawal was automatically measured by the apparatus. Paw withdrawal latencies were obtained using an infrared intensity of 70 W and measured three times separated by a minimum interval of 5 min. The average paw withdrawal latency was calculated from the three measurements. Minimum and maximum cutoffs were assigned at 1 and 30 s, respectively.

Measurement of estradiol levels in serum

Sixty-two hours after starting the examination, samples of venous blood (1.0 ml) were obtained from the right atrium. Serum estradiol levels were determined by the enzyme-linked immunosorbent assay (ELISA) sandwich method (Cayman Chemical, Ann Arbor, MI, USA). Monoclonal antibody specific to rat estradiol was precoated on 96-well plates. Serum samples, negative control, and diluted estradiol standard markers were added to the plates for analysis. Samples and standards were detected according to the manufacturer's instructions. Absorbance at 450 nm was recorded using an ELISA plate reader (model 680 Microplate Reader; Bio-Rad Laboratories, Hercules, CA, USA).

Intrathecal drug administration

Drugs were administered intrathecally 62 h following FCA treatment. Intrathecal yohimbine (5 mg/kg) and methysergide (1 mg/kg) were administered to the 1.0 mg/kg E_2 group. First, we performed an L3–L4 laminectomy under sevoflurane anesthesia. After the spinal cord was visualized, 10 μl of each solution was injected through a lumbar puncture between the L3 and L4 vertebrae using a 30-gauge tuberculin needle. Drug concentrations were chosen that have no significant effect on nociceptive threshold or behavior in rats based on dosages determined by Kawamura et al. [14, 15]. After administration of yohimbine or methysergide, we immediately stopped sevoflurane administration and evaluated withdrawal latency 1 h after administration of each drug.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analyses were carried out with StatView version 5.0 software (SAS Institute, Cary, NC, USA). Data were analyzed by repeated-measures and two-factor analysis of variance followed by Scheffe's post hoc test for multiple comparisons. P values <0.05 were considered significant.

Results

Estradiol effects on FCA-induced histological changes

Inflammatory cell infiltration and histological changes such as edema were observed in the right hind paw (i.e., that treated with FCA) of the sesame oil group compared to the left hind paw of the sesame oil group with saline (Fig. 2a–d). This histological change was also observed in the 1.0 mg/kg E_2 group with FCA to the right hind paw (Fig. 2e, f). Moreover, there was no significant improvement in inflammatory cell infiltration and histological changes between the sesame oil group with FCA to the right hind paw and the 1.0 mg/kg E_2 group with FCA to the right hind paw.

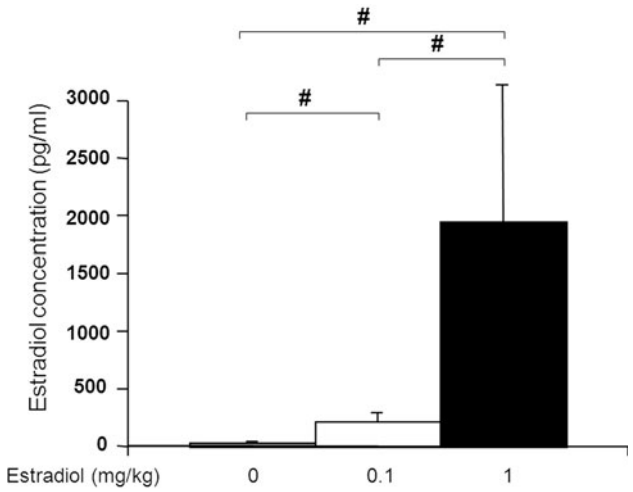

Fig. 3 Serum estradiol levels 62 h after FCA administration for sesame oil ($n = 6$; *gray bar*), 0.1 mg/kg E_2 ($n = 6$; *white bar*), and 1 mg/kg E_2 ($n = 6$; *black bar*) groups. Data are expressed as mean ± SD. $^{\#}P < 0.05$

Serum estradiol levels after estradiol administration

Serum estradiol concentration was within the normal range in the control group but was significantly elevated in the groups administered 0.1 and 1.0 mg/kg estradiol. In particular, estradiol concentrations increased to 2,000 pg/ml 62 h after administration of 1.0 mg/kg estradiol (Fig. 3).

Effect of estradiol on hyperalgesia

Withdrawal latency in the sesame oil group with saline was approximately 12 s during the entire observation period. Mean withdrawal latency in the sesame oil group with FCA was 4 s on the day following administration, gradually increasing and reaching 7 s by day 6. The 1.0 mg/kg E_2 group with FCA demonstrated significant increase in withdrawal latency on days 2 through 6 compared to the sesame oil group with FCA. In contrast, withdrawal latency for the 0.1 mg/kg E_2 group with FCA was not significantly different from that of the sesame oil group with FCA (Fig. 4). The 1.0 mg/kg E_2 group showed a similar latency as the sesame oil group with saline (data not shown).

Effects of naloxone on estradiol antinociceptive action

Naloxone significantly shortened withdrawal latency in the sesame oil group treated with FCA. In contrast, the analgesic effects of estradiol did not appear to depend on endogenous opioids, as naloxone was ineffectual in rats treated with 1 mg/kg E_2 (Fig. 5). Estradiol 1 mg/kg had no analgesic effects on the intact hind paw (Fig. 5).

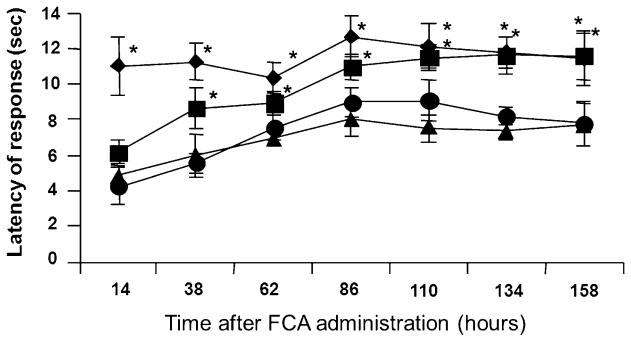

Fig. 4 Effect of estradiol treatment on withdrawal latency in the plantar test determined 62 h after FCA injection. *Diamonds*, sesame oil group with saline; *triangles*, sesame oil with FCA; *circles*, 0.1 mg/kg E_2 with FCA; *squares*, 1 mg/kg E_2 with FCA. Data are expressed as mean ± SD ($n = 6$–8 rats/group). $^{*}P < 0.05$ compared with the sesame oil group with FCA. E_2, estradiol

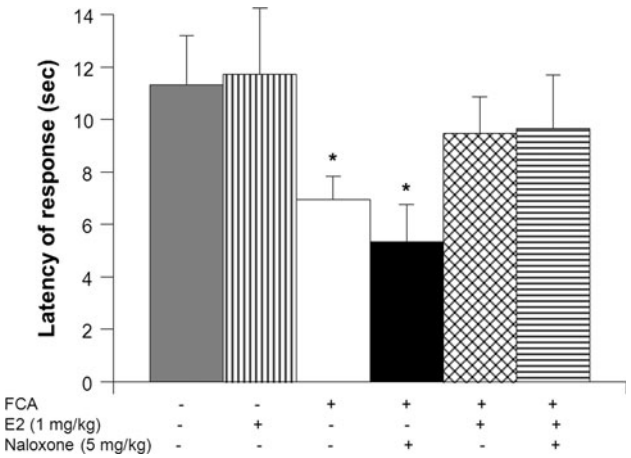

Fig. 5 Changes in paw withdrawal latency in the plantar test in rats treated with naloxone. Naloxone was administered 62 h after injection of FCA. Data are expressed as mean ± SD ($n = 6$). $^{*}P < 0.05$ compared with the 1 mg/kg E_2 group with FCA. E_2, estradiol

Effects of the α-2 receptor and serotonin system on estradiol antinociceptive effects

Yohimbine and methysergide attenuated the antinociceptive effects of estradiol (Fig. 6). In contrast, yohimbine and methysergide did not affect withdrawal latency in the 1 mg/kg E_2 group treated with saline (Fig. 6). In the sesame oil group with FCA, yohimbine and methysergide did not affect withdrawal latency (data not shown).

Discussion

The present study demonstrates that estradiol can reduce hyperalgesia, even when administered after the onset of hyperalgesia. Our findings further indicate that the

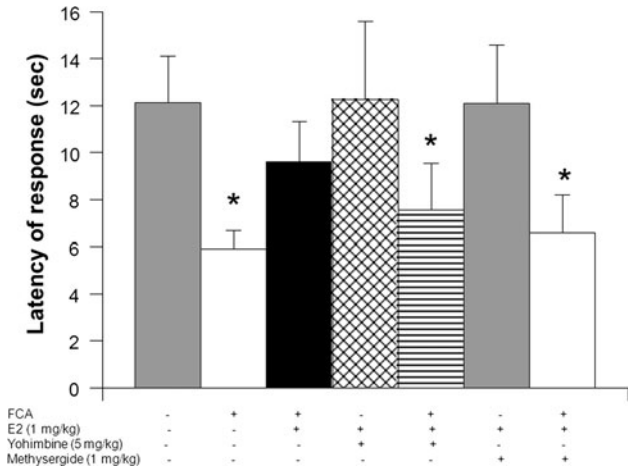

Fig. 6 Effects of drug treatment on withdrawal latency in the plantar test as determined 62 h after FCA-induced hyperalgesia. *Bar graph* shows the effects of intrathecal yohimbine (5 mg/kg) and methysergide (1 mg/kg) on FCA-induced hyperalgesia. Data are expressed as mean ± SD ($n = 6$). *$P < 0.05$ compared with 1 mg/kg E_2 group with FCA. E_2, estradiol

antinociceptive effect of estradiol involves α-2 and serotonin receptors, which suggests the possibility that estradiol may be used as an analgesic. On the other hand, estradiol showed no analgesic effect on the intact hind paw. Thus, estradiol may not influence normal pain states.

Recent studies have reported the existence of a descending pain inhibitory system as an endogenous analgesic mechanism [10, 11]. Many receptors are reported to be involved in this system, but the descending serotonin and noradrenaline pathways appear to be the main pathways [10, 11]. In the present study, estradiol effects were antagonized by α_2-adrenergic receptor antagonists, suggesting that one of the antinociceptive mechanisms of estradiol involves the descending noradrenaline pathway. The 5-HT receptor antagonist methysergide also attenuated the effects of estradiol, suggesting that the descending serotonin pathway is also involved. However, additional studies are needed to elucidate the role of the serotonin pathway in estradiol-mediated analgesia.

We also considered the possibility that endogenous opioid systems are an important element of the descending pain inhibitory system, as they have previously received attention as antinociceptive mediators. Previous studies in rats showed that endogenous opioids such as β-endorphin and methionine-enkephalin are released when inflammation occurs in tissue, activating opioid receptors and peripheral sensory ganglia, resulting in antinociceptive effects [9, 16]. In the current study, naloxone reversed the analgesic effects seen in rats treated with sesame oil vehicle and FCA. These results suggest that endogenous opioids have a physiological role in analgesic responses. In contrast, we found that naloxone had no effect on the antinociceptive effects of estradiol, in agreement with work by Stoffel et al. [17] that estradiol has no effect on opioid actions as they relate to antinociception. These findings lead us to conclude that endogenous opioid systems play no role in the antinociceptive effects of estradiol.

This study has some limitations. First, the effects of estradiol were tested only on male rats; estradiol may exert different effects on female rats. Second, we only evaluated the early analgesic effects of estradiol. Additional studies will be needed to test the efficacy of estradiol in other pain models and to determine the long-term efficacy of estradiol.

Our findings indicate that estradiol has antinociceptive effects, even after the onset of hyperalgesia. Further, we determined that the antinociceptive effects of estradiol on adjuvant-induced hyperalgesia in rats involve activation of adrenergic and serotonergic systems. Our findings demonstrate that estradiol has potential for use as a new analgesic.

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Conflict of interest The authors declare that they have no competing interests.

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