Pharmacology, Biochemistry and Behavior 98 (2011) 62–66

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Increase in formalin-induced tonic pain by 5alpha-reductase and aromatase inhibition in female rats

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ARTICLE INFO

Article history:
Received 27 September 2010
Received in revised form 10 December 2010
Accepted 14 December 2010
Available online 22 December 2010

Keywords:
5alpha-reductase
Aromatase
Morphine
Ovariectomy
Formalin test

ABSTRACT

Little is known about the role of steroidogenic enzymes in pain modulation. This study examined the effects of 5α-reductase and aromatase inhibition on formalin-induced tonic pain (FITP) in adult female rats. The animals received subcutaneous injection (5 mg/kg) of finasteride (an inhibitor of 5α-reductase) and letrozole (an inhibitor of aromatase), either separately or in combination, 15 min before formalin injection at a low (0.25%) and high (2.5%) concentration. Pretreatment with inhibitors increased FITP evoked by injection of 0.25% formalin, but they were not effective on 2.5% formalin pain. The enhancing effects of finasteride and letrozole on FITP induced by 2.5% formalin was demonstrated by inhibitory actions of these drugs on morphine (7 and 10 mg/kg, intraperitoneal) induced antinociception. The nervous system could be considered as the main target of the enzymes inhibition, since the pronociceptive effect was also observed after administration of inhibitors to ovariectomized rats. Altogether, these findings suggest that the biological activity of the enzymes 5α-reductase and aromatase modulates FITP and may help to develop effective therapeutic strategies to counteract pain.

1. Introduction

There is considerable evidence suggesting that steroidal hormones influence pain processing (Fillingim and Ness, 2000; Aloisi and Bonifazi, 2006). In recent years, apart from peripheral steroids, these studies have also been directed toward neuroactive steroids which are de novo synthesized in the brain, spinal cords (Mensah-nyagan et al., 2008) and sensory nerves (Schaeffer et al., 2010), and may have potential therapeutic use for the management of some types of pains that may not respond well to current pharmacological approaches (Gasior et al., 1999; Herd et al., 2007; Aouad et al., 2009). Both metabolism and biosynthesis of steroids occur in the brain and spinal cord which express the enzymes required for the sequential conversion of cholesterol into pregnan steroids (Paul and Purdy, 1992; Compagnone and Mellon, 2000; Stoffel-Wagner, 2001, 2003). The mechanism of enzymatic regulation is still under investigation and recent findings in female (Aydin et al., 2008; Pradhan et al., 2008) and male (Pradhan et al., 2008) rats reveal that steroidogenic enzymes such as aromatase or 3β-hydroxysteroid dehydrogenase can be acutely regulated through mechanisms independent of protein transcription. It has been shown that neuropathic pain increases activity of cytochrome P450 side-chain cleavage resulting in an increase in spinal cord concentrations of allopregnanolone which strongly stimulates type A receptors for GABA, a pivotal neurotransmitter involved in pain modulation (Patte-Mensah et al., 2004, 2006). The biological activity of 3α-hydroxysteroid oxide-reductase (3α-HSOR) in the rat spinal cord is up-regulated after sciatic nerve injury which increases thermal and mechanical pain threshold (Meyer et al., 2008). In addition, women treated with aromatase inhibitors like letrozole often experience arthralgesia which in some cases, has necessitated discontinuation of treatment, leading to prompt relief of symptoms (Felson and Cummings, 2005; Coleman et al., 2008). Taken together, the results of these studies suggest a direct link between clinical pain and neuroactive steroid formation in the nervous system, but there is a pressing need for more animal studies to clarify the role of steroidogenic enzymes in pain processing which may open up new perspectives for the treatment of pain.

The formalin test is widely used to study pain (Fu et al., 2001; Tjolsen et al., 1992; Watson et al., 1997). The long lasting painful stimuli in the second phase of the formalin test may be more analogous to pain in people (Aloisi et al., 1998) and is thought to reflect tonic pain associated with inflammation and the brainstem plays a critical role in its generation (Matthies and Franklin, 1992). Previously, we reported that formalin-induced tonic pain (FITP) sharply decreases brain and spinal cord concentration of testosterone through an increase in 5α-reductase (5α-R) activity (Amini and Ahmadiani, 2002). These findings raise the question of the importance of 5α-R activity in FITP. Since we have observed that pretreatment with finasteride (an inhibitor of 5α-R) prevents FITP-induced depletion of the brain and spinal cord testosterone (Amini and

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0091-3057/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2010.12.016

Ahmadiani, 2002), a possible shift to the aromatase pathway should be considered. Aromatase converts testosterone to estradiol. Its activity is well documented in the CNS and the relevance of local estrogen synthesis for pain processes has been reported (Evrard and Balthazart, 2004; Evrard, 2006). It has been suggested that the inhibition of the 5α-R pathway may increase the availability of testosterone to the aromatase pathway (Ribeiro and Pereira, 2005). This hypothesis is supported by the observation that either administration of finasteride (Ladle et al., 1997) or mutation of the 5α-R gene (Mahendroo et al., 1997) increases plasma estradiol levels. It has been reported that testosterone and estradiol do not significantly affect behavioral responses during the first phase of formalin test (Nayebi and ahmadiani, 1999; Kuba et al., 2006), but flutamide, an androgen receptor antagonist (Nayebi and Ahmadiani, 1999; Nayebi and Rezazadeh, 2004), and estradiol (Kuba et al., 2006) decrease severity of FITP. Accordingly, it could be suggested that effective inhibition of testosterone metabolism requires simultaneous blocking of 5α-R and aromatase pathways.

The present study was undertaken to determine the effects of finasteride and letrozole, administered either separately or in combination, on FITP using low concentration of formalin (i.e. 0.25%). In addition, morphine was used to evaluate possible pronociceptive effects of these drugs at high concentration of formalin (i.e. 2.5%). The pronociceptive effects of the enzymes inhibition was also investigated in ovariectomized rats to exclude ovaries as a main peripheral source of steroids and show the nervous system as the main target of the enzymes inhibition.

2. Methods

2.1. Animals

Experiments were performed on healthy female Wistar rats prepared from Pasteur Institute of Iran (Tehran, Iran). The rats were housed in groups of 5–7 per cage in the animal facility with a light–dark cycle of 13:11 h (lights on at 06.00 h and off at 19.00 h) and temperature (20 ± 2 °C) with ad libitum access to food (pellet from Pars Co., Tehran, Iran) and natural mineral water. The rats were allowed to habituate to the housing facilities prior to being randomly assigned to behavioral studies. They were used at a weight range of 170–220 g without estrous cycle determination. The number of animals for each group of experiments was 6 and each animal was tested only once. Experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985) and had approval from the deputy of research of the university. Efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs and administration protocols

Finasteride and letrozole were obtained from Sigma (St. Louis, MO, USA). These drugs were dissolved in 20% ethanol in sesame oil and administered subcutaneously (s.c.) at a dose of 5 mg/kg in a volume of 1 ml/kg. Finasteride (Celotti et al., 1997; Amini and Ahmadiani, 2002) and letrozole (Aydin et al., 2008) were used at doses that could inhibit the CNS 5α-R and aromatase. These drugs were administered 15 min before formalin injection to decrease possible shift of endogenous testosterone to aromatase or 5α-R pathways. Morphine sulfate was prepared from Temad Co. (Tehran, Iran). It was dissolved in saline and administrated interaperitoneally (i.p.) at doses of 5, 7 and 10 mg/kg in a volume of 1 ml/kg, 15 min before formalin injection.

2.3. Ovariectomy

The female rats were ovariectomized under xylazine (12 mg/kg, i.p.; Alfasan, Woerden, Holland) and ketamine (60 mg/kg, i.p.; Rotexmedica, Trittau, Germany) anesthesia. A small incision (1 cm) was made through the skin and the muscle back walls in parallel with the body line. The ovaries were then located, and a silk thread was tightly tied around the oviduct, including the ovarian blood vessels. The oviduct was sectioned, and the ovary was removed, taking good care in leaving the knot intact. The skin and the muscle wall were then sutured with a silk thread. A drop of 0.25% bupivacaine (Merck Generique, France) was placed on the incision site at the time of closure. Pain behavioral studies were performed 2 weeks after ovariectomy.

2.4. Formalin test

Animals were placed in a formalin test chamber for adaptation 2 h before testing. A volume of 50 μl diluted formalin (a 37% solution of formaldehyde was diluted with saline) was injected s.c. in the plantar pad of the right hind paw. The gauge of the needle was 27. To obtain stable scores from low formalin concentrations, it is necessary to ensure that the needle is inserted through the skin and run for 5 mm under the skin. Formalin injection was carried out to induce nociceptive responses and immediately after injection, the animal was placed in the formalin chamber (a clear plastic 40 × 40 × 40 cm). For a better view of the animals' paws a mirror at a 45° angle was placed underneath. Specific pain-related behaviors is visually observed until 45 min after injection and a continuous record of how the animal treated the injected paw, with full weight bearing scoring '0', light pressure on the floor or limping '1', elevation of the paw '2' and licking or biting the paw '3' (Dubuisson and Dennis, 1977). Immediately, at the end of the formalin test, the animal was killed by deep anesthesia.

2.5. Statistical analysis

Data were analyzed as the mean of pain score (±SEM) during Phase II (20–45 min) by one-way analysis of variance (one-way ANOVA). In the case of significant variation, the values were compared by Tukey–Kramer multiple comparisons test. Statistical significance was accepted at the level of $P<0.05$.

3. Results

3.1. Nociceptive behavior evoked by different concentrations of formalin

The injection of different concentrations of formalin (0.25, 0.5, 1, and 2.5%) into the plantar surface of the rat hind paw evoked the typical biphasic nociceptive responses, consisting of an early excitatory phase lasting 5 min after injection and a prolonged second phase lasting from about 20 to 45 min which was considered as FITP. Pain behaviors in the first phase were not related to formalin concentration and there were no effects of treatments. The first phase is not discussed further. Injection of saline did not produce any significant pain behaviors on FITP. Mean pain score (±SEM) in FITP following injection of 0.25, 0.5, 1 and 2.5% formalin were 0.94 (0.124), 1.96 (0.07), 2.44 (0.07) and 2.46 (0.059), respectively. Analysis of variance [$F(3,20)=66.06$, $P<0.0001$] revealed a significant difference between groups. Injection of formalin at 0.25 and 0.5% concentrations produced lower pain behaviors than 2.5% formalin (Fig. 1).

3.2. Effects of finasteride and letrozole on FITP

The experiments assessing of the effect of finasteride and letrozole, administered either separately or in combination, on nociceptive responses after injection of 0.25% formalin [$F(3,20)=31.45$, $P<0.0001$] showed a significant difference between groups. The effects of finasteride and letrozole were significant after single and combined administration (Fig. 2A). There was no difference between

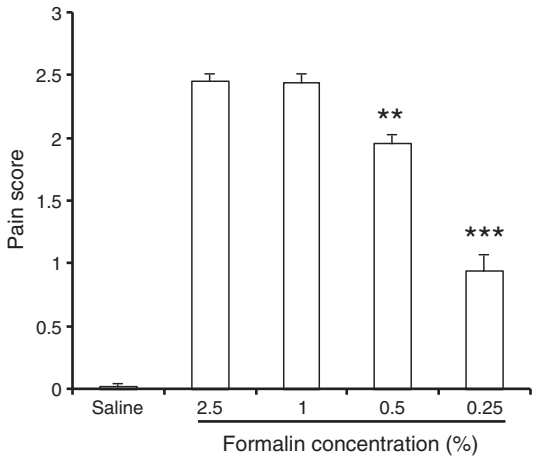

Fig. 1. The effect of formalin (0.25–2.5%) or saline injection (50 μl) into the plantar pad of the right hind paw of the adult female rats. Each bar represents the mean ± S.E.M. of pain score during 20–45 min (late phase) after formalin injection (n = 6 per group). One-way ANOVA followed by Tukey–Kramer multiple comparisons test. The saline group was not included in comparison. $^{**}P<0.01$, $^{***}P<0.001$ when compared with 2.5% formalin group.

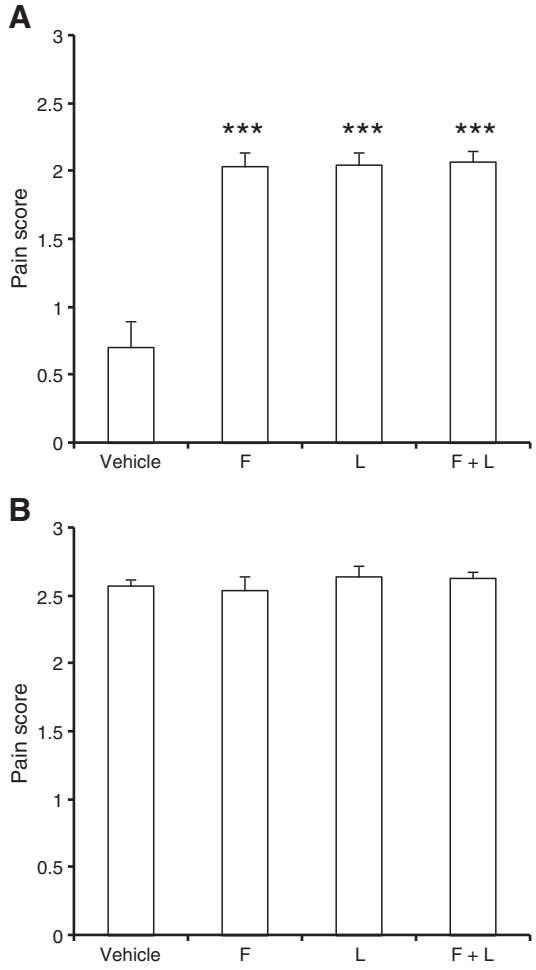

Fig. 2. The effects of pretreatment with finasteride (F), letrozole (L), finasteride + letrozole (F + L) at a dose of 5 mg/kg or vehicle of inhibitors (20% ethanol in sesame oil) on nociception threshold after injection of 0.25% (Fig. A) or 2.5% formalin (Fig. B) in adult female rats. Inhibitors were administered 15 min before formalin injection. Each bar represents the mean ± S.E.M. of pain score during 20–45 min (late phase) after formalin injection (n = 6 per group). One-way ANOVA followed by Tukey–Kramer multiple comparisons test. $^{***}P<0.01$ when compared with vehicle group using 0.25% formalin injection. $P>0.05$ when F or L group compared with F + L group.

the effects of finasteride or letrozole or the combination of both. Nociceptive responses after administration of inhibitors using 2.5% formalin [$F(3,20) = 0.38$, $P = 0.77$] did not reveal a significant difference between treatment groups (Fig. 2B).

3.3. Effects of finasteride and letrozole on morphine-induced analgesia using 2.5% formalin injection

We hypothesized that the pronociceptive effects of finasteride and letrozole is masked when 2.5% formalin injection is applied. Therefore, we used morphine to demonstrate the pronociceptive effects of the enzymes inhibition even using 2.5% formalin. Pain behaviors in FITP following i.p. injection of saline or morphine at doses of 5, 7 and 10 mg/kg [$F(3,20) = 23.13$, $P<0.0001$] showed a significant difference between treatment groups. Morphine produced analgesia at doses of 7 and 10 mg/kg (Fig. 3).

The effects of pretreatment with finasteride and letrozole on two effective analgesic doses of morphine in FITP were evaluated and significant differences were found in groups treated with 7 [$F(3,20) = 8.39$, $P<0.0008$] (Fig. 4A) and 10 mg/kg [$F(3,20) = 9.66$, $P<0.0004$] (Fig. 4B) morphine. Morphine analgesia at doses of 7 and 10 mg/kg was attenuated by single or combined administration of letrozole and finasteride at both doses. There was no difference between the finasteride or the letrozole group and the group that received both drugs in the morphine 7 and 10 mg/kg studies.

3.4. Effects of finasteride and letrozole on FITP in ovariectomized rats

Nociceptive responses after administration of finasteride and letrozole in low concentration of formalin (0.25%) in ovariectomized rats were investigated and analysis of variance [$F(4,25) = 26.94$, $P<0.0001$] revealed a significant difference between groups. Nociceptive responses after single or combined administration of finasteride and letrozole were increased significantly (Fig. 5). There was a statistical difference between the finasteride group and the group that received both drugs.

4. Discussion

As expected (Munro, 2009), behavioral response of formalin injection at low concentrations (0.25 and 0.5%) were significantly lower than 2.5% formalin. Animals that were pretreated with

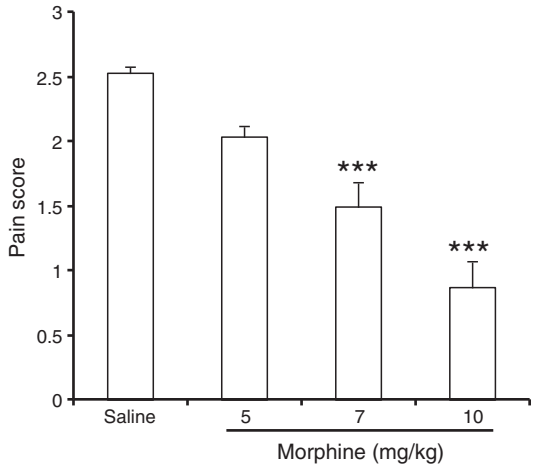

Fig. 3. The effects of administration of morphine on nociception threshold of 2.5% formalin in adult female rats. Morphine (5, 7 and 10 mg/kg, i.p.) was administered 15 min before formalin injection. Each bar represents the mean ± S.E.M. of pain score during 20–45 min (late phase) after formalin injection (n = 6 per group). One-way ANOVA followed by Tukey–Kramer multiple comparisons test. $^{***}P<0.001$ when compared with vehicle (saline) group.

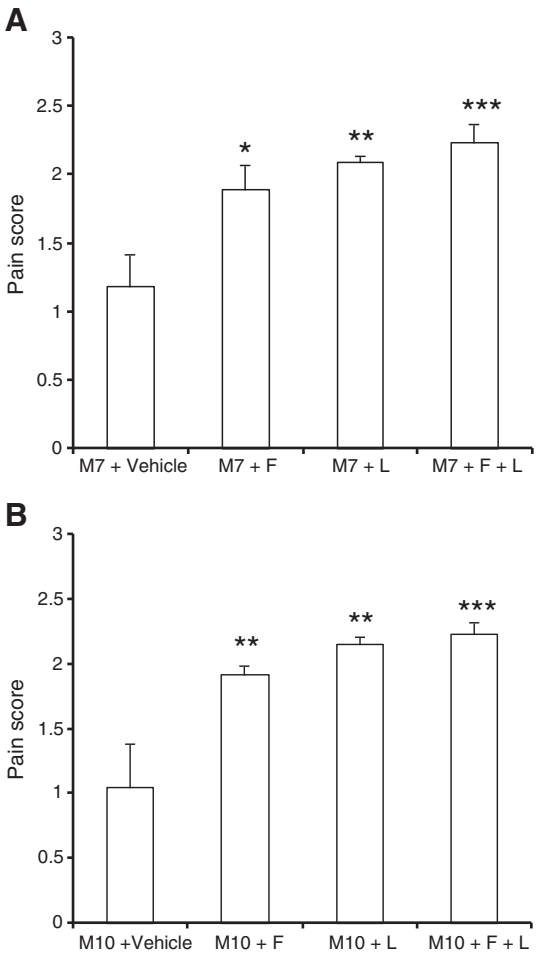

Fig. 4. The effects of pretreatment with finasteride (F), letrozole (L) and finasteride + letrozole (F + L) and vehicle of inhibitors (20% ethanol in sesame oil) on morphine-induced analgesia in adult female rats. Inhibitors (5 mg/kg) and morphine at doses of 7 (M7, Fig. A) or 10 mg/kg (M10, Fig. B) were administered 15 min before 2.5%formalin injection. Each bar represents the mean ± S.E.M. of pain score during 20–45 min (late phase) after formalin injection (n = 6 per group). One-way ANOVA followed by Tukey–Kramer multiple comparisons test. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ when compared with M + vehicle group. $P>0.05$ when F or L groups compared with corresponding F + L group.

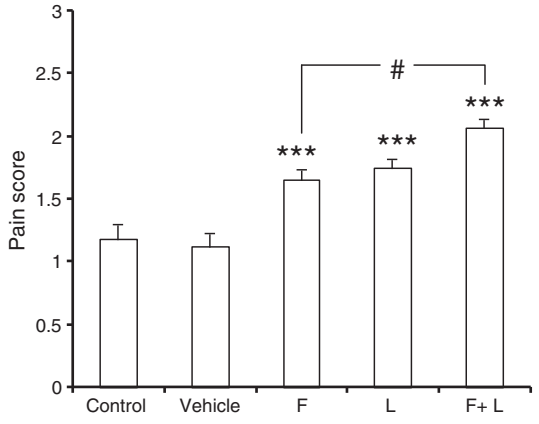

Fig. 5. The effects of pretreatment with finasteride (F), letrozole (L), finasteride + letrozole (F + L) or vehicle of inhibitors (20% ethanol in sesame oil) on nociception threshold in ovariectomized rats. Inhibitors (5 mg/kg) were administered 15 min before 0.25% formalin injection. Each bar represents the mean S.E.M. of pain score during 20–45 min (late phase) after formalin injection (n = 6 per group). One-way ANOVA followed by Tukey–Kramer multiple comparisons test. $^{***}P<0.001$when compared with vehicle group. $\#P<0.05$ when F group compared with F + L group.

inhibitors were significantly more sensitive to the nociceptive effect of 0.25% formalin than the vehicle-injected animals. However, these hyperalgesic effects were not observed using 2.5% formalin injection. It could be that 2.5% formalin produces saturation of the behavioral pain response. Inhibitors plus 2.5% formalin probably produce more pain, but there is a limit to how much of the time a rat can elevate or lick its paw, so the score would be no higher. Thereby, the hyperalgesic effects of inhibitors are masked by 2.5% formalin. In the present work, the hyperalgesic effects of finasteride and letrozole in FITP produced by 2.5% formalin was demonstrated by attenuation of the antinociceptive effects of morphine. The enzyme inhibitors increase pain, and when pain is at or above the behavioral ceiling, morphine reduces the level into the active range of the formalin concentration–effect relationship.

We may assume that the observed effects of letrozole and finasteride in the present study resulted from their local actions in the nervous system. However, the two enzymes blocked in this study are not only present in the nervous system and they have been described in several other tissues. It should be stressed that the female rats in the present study were ovary-intact and the stage of the estrous cycle was not assessed. This, as well as the systemic administration of drugs, makes it possible that the actions of the drugs were peripheral, particularly in the ovary. Recent studies have shown that peripheral estradiol induces temporomandibular joint antinociception in rats (Favaro-Moreira et al., 2009), which is affected by the estrous cycle (Fischer et al., 2008). Therefore, in the present study, ovariectomy was performed to eliminate the main peripheral source of the steroids. The results of our study revealed that inhibitors increase pain-related behaviors in ovariectomized rats. Accordingly, the nervous system could be considered as the main target of enzymes inhibition.

Although, the interactions between the two enzymatic pathways were apparent in ovariectomized groups, the present study does not provide strong evidence that these drugs have additive effects. Inhibitors are both given at doses that probably saturate a single mechanism, but in lack of a full dose–response curve, it cannot be determined if the effects are additive or not.

We have reported that following formalin injection, testosterone is sharply decreased in the brain and spinal cord through increased metabolism via 5α-R pathway (Amini and Ahmadiani, 2002). Two isozymes of 5α-R have been identified in different brain regions (Sanchez et al., 2008) and it has been found that 5α-R1 is by far the most abundant molecular form in the brain of rat, mouse and human (Thigpen et al., 1993; Stoffel-Wagner, 2003; Torres and Ortega, 2003). For both isozymes, progesterone is the preferred substrate, followed by testosterone and at a considerable distance corticosterone. In humans, finasteride is a week inhibitor of 5α-R1 and a good inhibitor of 5α-R2, but it can inhibit both isozymes in rats (Occhiato et al., 2004). In the brain, testosterone is readily metabolized by 5α-R to dihydrotestosterone, which is then reduced by 3α-hydroxysteroid oxide-reductase (3α-HSOR) to 3α-androstanediol (3α-diol) (Celotti et al., 1997; Kellogg and Frye, 1999; Frye et al., 2001). 3α-diol, produced de novo in the brain (Martini et al., 1996), is a very effective modulator of GABA/benzodiazepine receptor complexes, and has analgesic properties (Frye et al., 1996). Besides testosterone, finasteride also inhibits a whole cascade of progesterone metabolites, of which 3α, 5α tetrahydroprogesterone (allopregnanolone) and 3α, 5β tetrahydroprogesterone (pregnanolone) are both potent $GABA_A$ agonists and capable of producing analgesia in formalin test (Ocvirk et al., 2008). Therefore, finasteride may prevent 3α-diol, allopregnanolone and pregnanolone production during FITP and increase the feeling of pain. Enhancing FITP by 5α-R inhibition is in line with recent findings of a pivotal role of 3α-HSOR in neuropathic pain (Meyer et al., 2008) and local adjustment of the strength of synaptic inhibition in spinal cord by controlling the synthesis of endogenous 5α-reduced neuroactive steroids (Keller et al., 2004; Poisbeau et al., 2005). Similarly, it has been shown that 5α-reduced neuroactive steroids are up-regulated in spinal cord during

inflammatory pain, reducing thermal heat hyperalgesia (Schlichter et al., 2006).

The results of the present study also revealed the importance of aromatase activity in FITP. Estradiol has been shown to be analgesic in a model of neuropathic (Tsao et al., 1999) or formalin pain (Kuba et al., 2006). Therefore, the basis for the effects of letrozole could be reduction in estrogen synthesis.

To the best of our knowledge, the present data provide the first report of the behavioral effects of 5α-R and aromatase inhibition in FITP. Altogether, these results suggest that 5α-R and aromatase are involved in pain processing and could be a cellular target for drugs. These results may also help to develop effective therapeutic strategies against inflammatory pain.

Acknowledgements

This study was supported by a grant from Deputy of Research, Golestan University of Medical Sciences. We would like to thank Seyed Yaghoub Jafari, Deputy of Culture and Student for his kind support and Dr. Ghafouri (Iran Hormone Pharmaceutical Company, Tehran, Iran) for donating letrozole.

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