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Involvement of serotoninergic mechanism in analgesia by castration and flutamide, a testosterone antagonist, in the rat formalin test

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Abstract

Several studies have suggested that testosterone has a role in nociception. Recently, we have shown that castration and flutamide, a testosterone antagonist, induce analgesia in the late phase of formalin test, which is related to increase of 5-HT levels in the dorsal horn of the lumbar spinal cord. The aim of the present study was to investigate the effect of fluoxetine, a selective serotonin reuptake inhibitor, on castration and flutamide-induced analgesia in order to further explore the role of 5-HT systems in such analgesia. Four weeks after castration, there was an analgesia in the late phase of formalin test, and this was potentiated by acute (0.32 mg kg^{-1} ip) treatment of fluoxetine. Furthermore, coadministration of fluoxetine (0.32 mg kg^{-1} ip) and flutamide (10 mg kg^{-1} ip) produced more antinociceptive effect than those animals receiving fluoxetine and flutamide alone. The analgesic effect of fluoxetine (0.32 mg kg^{-1} ip) and flutamide (10 mg kg^{-1} ip) was abolished by pretreatment with 5,7-DHT (100 μg/rat it) and naloxone (2 mg kg^{-1} ip). In summary, our data suggest that fluoxetine and flutamide have antinociceptive effects in tonic inflammatory pain through functional alteration of serotonergic systems, and their effects are potentiated by coadministration. The possible role of opioidergic system in their antinociceptive effect cannot be neglected.

Keywords: Fluoxetine; Castration; Flutamide; Formalin test

1. Introduction

The formalin test is a valuable method available to induce a long-lasting noxious input towards the spinal cord and the CNS (Tjolsen et al., 1992). The subcutaneous injection of formalin into the rat hindpaw produces a biphasic excitatory-evoked behavioral response (Dubuisson and Dennis, 1977). The formalin test is different from most models of pain in that it may induce a state with good approximation to some clinical conditions of chronic inflammatory pain.

The dorsal horn of the spinal cord is innervated by serotoninergic neurons, which are involved in the modulation of nociceptive transmission (Tonyl et al., 1979; Abhold and Bowker, 1990; Wang and Nakai, 1994). Hyperalgesia following administration of 5-HT receptor antagonists or the lesion of the raphe-spinal 5-HT system has been reported in the tail-flick and hot-plate tests (Fasmer et al., 1985). Activation of descending bulbospinal neurons by electrical stimulation of the nucleus raphe magnus promotes the release of 5-HT and increases its turnover in the dorsal horn of the spinal cord (Rivot et al., 1982; Hammond et al., 1989). Serotonin 5-HT_3 and 5-HT_{1A} receptors have been identified in the dorsal horn of the spinal cord subserving nociception and analgesia, which suggests an essential role for serotonin in pain modulation (Tonyl et al., 1979; Oyama et al., 1996; Bardin et al., 1997, 2000). Recently, we have shown that chronic and acute administration of fluoxetine, a selective serotonin reuptake inhibitor, produces antinociceptive effects in tonic inflammatory pain through functional alteration of the serotoninergic system (Nayebi et al., 2001).

A number of observations indicate that testosterone can play a significant role in nociception. There is hypoalgesia using thermal algesic tests in castrated rats (Rao and Saifi, 1981). Leuprolide (a slow-release gonadotropin-releasing hormone analogue) can cause a significant reduction in the severity of cluster headaches and has been postulated to produce its benefit by lowering testosterone serum levels (Nicolodi et al., 1993). According to another study, cas-

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tration reduces both opioid and nonopioid swim stress-induced analgesia in rats, which was reversed by testosterone replacement (Romero et al., 1988).

There are many reports on testosterone interactions with serotoninergic systems. It has been reported that castration increases the biosynthesis of 5-hydroxytryptophan in limbic forebrain and diencephalon of the rat (Long et al., 1983). Evidence from another study shows that androgens interact with serotonin to control sexual dimorphisms in the rat lumbosacral spinal cord (Cowburn and Payne, 1994). Others have suggested that in men with secondary hypogonadism, there is an increase in urinary 5-hydroxyindoleacetic acid (5-HIAA), which responds to testosterone therapy (Shakir et al., 1996). Previously, we reported the involvement of spinal serotoninergic systems in the influence of testosterone on formalin-induced pain by using spinal microdialysis technique. In that study, we showed that decreasing testosterone levels (i.e., castration) or blocking testosterone receptors with flutamide (a testosterone antagonist) produced analgesia in the late phase of the formalin test, and this correlated with the increase of 5-HT levels in the dorsal horn of the spinal cord (Nayebi and Ahmadiani, 1999). The aim of present study was to investigate the interactions between fluoxetine, which inhibits 5-HT uptake, and castration and flutamide in order to further examine the involvement of central 5-HT pathways in their actions.

2. Materials and methods

2.1. Animals

The experiments were carried out on male Wistar rats weighing 275–300 g. Animals were housed in standard polypropylene cages, six per cage, under a 12:12-h L/D schedule at an ambient temperature of 23 ± 2 °C and were allowed food and water. Following surgical implantation of intrathecal cannula, the animals were housed individually in each cage to avoid possible displacement or disruption of the cannula. Experiments were carried out in accordance with the guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No 85-23, revised 1985.)

2.2. Drugs

All chemicals and biochemicals were obtained from Sigma (USA) except for testosterone enanthate, which was purchased from Daroo Pakhsh (Tehran, Iran).

Solutions were prepared fresh on the days of experimentation. Fluoxetine, flutamide, testosterone enanthate, naloxone and neurotoxin, 5,7-dihydroxytryptamine creatinin sulfate (5,7-DHT) were dissolved in ethanol–water (1:10, v/v), ethanol–water (2:1, v/v), sterile castor oil, 0.9% saline and 0.9% saline containing 0.2 mg ml^{-1} ascorbic acid, respectively. The 5,7-DHT (100 μg/rat) was administered intrathecally 5 days before the experiment. Desipramine (10 mg kg^{-1}) was administered intraperitoneally 30 min before the 5,7-DHT administration to prevent uptake of 5,7-DHT into catecholaminergic neurons and subsequent damages. Drugs were administered in a volume of 10 μl on intrathecal route 5 min before formalin injection. The intraperitoneal administrations of drugs were done 30 min before the start of experiments. In order to replace testosterone in castrated rats, testosterone enanthate was injected intraperitoneally on fourth week of castration for 3 days.

2.3. Surgical procedures

The animals were anaesthetized by intraperitoneal injection of sodium thiopental (50 mg kg^{-1}). Castration was performed by exposing the testes via bilateral midscrotal incisions and crushing the vas deferens with a hemostat, then the testes and testicular fat were removed. The sham surgery consisted of exposing the gonads without removing them. Pain behavioral studies were performed 4 weeks after castration. For intrathecal administration, animals were cannulated intrathecally with a PE-10 catheter inserted caudally 8.5 cm from the atlanto-occipital membrane (Yaksh and Rudy, 1976).

2.4. Formalin test

The rats were placed in a quiet room during the light phase of the light–dark cycle. Before formalin injection, the rats were placed individually in a transparent cage (40 × 25 × 20 cm) and were left there at least for 30 min. After adaptation, 50 μl of diluted formalin 2.5% was injected subcutaneously into the plantar region of the hind paw for noxious stimulation. Pain rating was recorded according to the following behavioral categories: 0—weight is born evenly on both rear paws, 1—limps during locomotion or rests with injected paw favored, 2—injected paw is elevated with, at most, the nail touching the floor, and 3—injected paw is groomed or bitten (Dubuisson and Dennis, 1977). Rats were observed every 15 s during 20–60 min (late phase) after formalin injection. The obtained data were averaged for each individual and then were combined across individuals. The observer was blind to treatments.

2.5. Expression of data and statistics

Descriptive statistics and comparisons of differences between means of data sets were calculated by use of InStat software. The data were expressed as mean ± S.E.M. The significant differences between treatment conditions in each phase were first analyzed by the Kruskal–Wallis nonparametric analysis of variance test. Statistical significance was accepted at the level of $P < .05$. In the case of significant variation ($P < .05$), the values were compared by Dunn's multiple comparisons test.

3. Results

3.1. Effect of castration and testosterone replacement therapy on analgesic test

Due to lack of marked differences, the early phase data are not shown in any figures. Nonparametric analysis of variance revealed a significant interaction between groups in the late phase of formalin test (Kruskal–Wallis, $P<.0001$) (Fig. 1). Further analysis showed that pain sensitivity was different ($P<.05$) in 4-week castrated rats when compared with sham-operated or intact rats. This analgesic effect was reversed by replacement therapy with testosterone enanthate (1 mg kg^{-1} for 3 days) in comparison with the 4-week castrated group.

3.2. Effect of acute administration of fluoxetine on castration-induced analgesia

Fig. 2 shows the significant difference between groups in the late phase (Kruskal–Wallis, $P<.0001$) (Fig. 2). Castration-induced analgesia was increased significantly ($P<.05$) by acute administration of fluoxetine (0.32 mg kg^{-1} ip).

3.3. Effect of acute administration of fluoxetine and flutamide on formalin-induced pain

Pain sensitivity was significantly different (Kruskal–Wallis, $P<.0001$) between groups in the late phase (Fig. 3). The acute intraperitoneal injection of both fluoxetine (0.32 mg kg^{-1}) and flutamide (10 mg kg^{-1}) produces analgesia in the formalin test. The antinociceptive effects of these drugs were potentiated ($P<.05$) after coadministration.

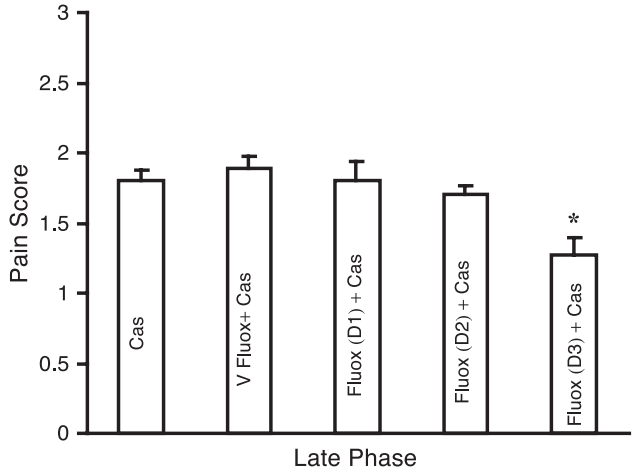

Fig. 2. The effect of acute administration of fluoxetine (D1 = 0.08, D2 = 0.16 and D3 = 0.32 mg kg^{-1} ip) on castration-induced analgesia. Each bar represents the mean ± S.E.M. of pain score during 20–60 min (late phase) after formalin injection ($n=6$ per group), Kruskal–Wallis nonparametric analysis of variance followed by Dunn's multiple comparisons test; *$P<.05$ when compared with sham-operated or intact rats. Cas = castration, V Fluox = vehicle of fluoxetine, Fluox = fluoxetine.

3.4. Effect of 5,7-DHT and naloxone on fluoxetine and flutamide-induced analgesia

Pain responses were significantly different between groups in the late phase (Kruskal–Wallis, $P<.0001$) (Fig. 4). In groups of animals treated acutely with fluoxetine (0.32

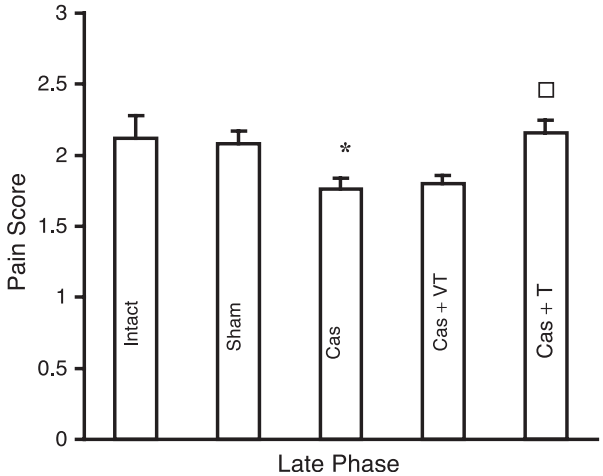

Fig. 1. The effect of castration and testosterone (1 mg kg^{-1} ip for 3 days) replacement therapy on formalin behavior. Each bar represents the mean ± S.E.M. of pain score during 20–60 min (late phase) after formalin injection ($n=6$ per group), Kruskal–Wallis nonparametric analysis of variance followed by Dunn's multiple comparisons test; *$P<.05$ when compared with sham-operated or intact rats, □$P<.05$ when compared with castrated group. Cas = castration, VT = vehicle of testosterone, T = testosterone.

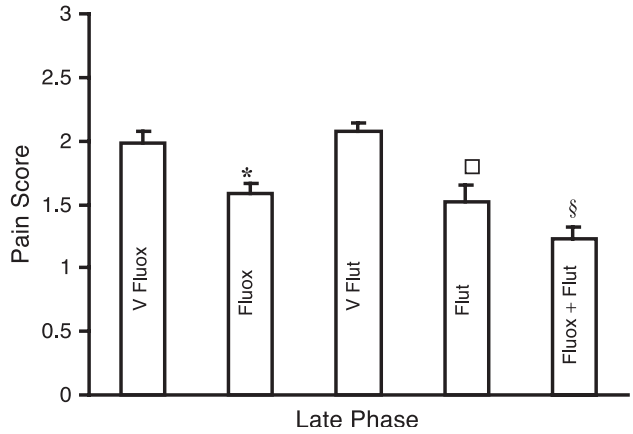

Fig. 3. The effect of fluoxetine (0.32 mg kg^{-1} ip), flutamide (10 mg kg^{-1} ip) and their combination on formalin-induced pain. Each bar represents the mean ± S.E.M. of pain score during 20–60 min (late phase) after formalin injection ($n=6$ per group), Kruskal–Wallis nonparametric analysis of variance followed by Dunn's multiple comparisons test; *$P<.05$ when compared to fluoxetine vehicle-treated rats, □$P<.05$ when compared to flutamide vehicle-treated rats, §$P<.05$ when compared with fluoxetine and or flutamide-treated rats. V Fluox = vehicle of fluoxetine, Fluox = fluoxetine, V Flut = vehicle of flutamide, Flut = flutamide.

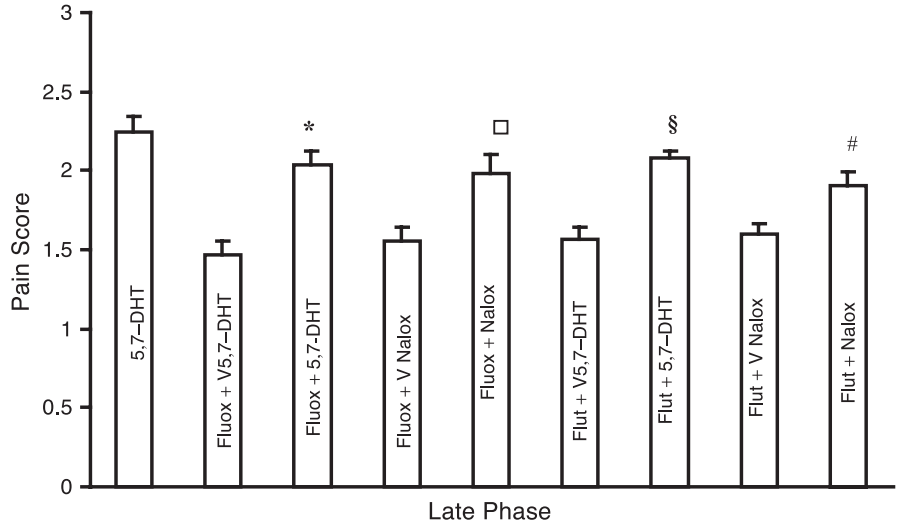

Fig. 4. The effect of fluoxetine (0.32 mg kg^{-1} ip) and flutamide (10 mg kg^{-1} ip) in 5,7-DHT (100 μg/rat it) and naloxone (2 mg kg^{-1} ip) pretreated rats on formalin-induced pain. Each bar represents the mean ± S.E.M. of pain score during 20–60 min (late phase) after formalin injection (n = 6 per group), Kruskal–Wallis nonparametric analysis of variance followed by Dunn's multiple comparisons test; * P < .05 when compared with fluoxetine administration in 5,7-DHT vehicle-treated rats, □ P < .05 when compared to fluoxetine in naloxone vehicle-treated rats, § P < .05 when compared to flutamide in 5,7-DHT vehicle-treated rats, # P < .05 when compared to flutamide in naloxone vehicle-treated rats. 5,7-DHT = 5,7-dihydroxytryptamine, Fluox = fluoxetine, V 5,7-DHT = vehicle of 5,7-DHT, V Nalox = vehicle of naloxone, Nalox = naloxone, Flut = flutamide.

mg kg^{-1} ip), analgesia was reversed markedly (P < .05) by pretreatment with both 5,7-DHT (100 μg/rat it) and naloxone (2 mg kg^{-1} ip). Furthermore, the analgesic effect of intraperitoneally administered flutamide (10 mg kg^{-1}) was reversed significantly (P < .05) by pretreatment with of 5,7-DHT (100 μg/rat it) and naloxone (2 mg kg^{-1} ip).

4. Discussion

The aversive response elicited by intraplantar injection of formalin is composed of two phases. The early phase seems to be caused predominantly by direct activation of C-fibres, while the late phase is dependent on the combination of an inflammatory reaction in the peripheral tissues and changes in the dorsal horn functions of the spinal cord. The late phase, expressed 20 min after formalin injection, is considered to be more useful than the early phase in the evaluation of drugs employed clinically in the treatment of inflammatory pain (Dubuisson and Dennis, 1977; Shibata et al., 1989; Junyi et al., 2001). Therefore, the late phase is more interesting to us than the early phase. Previously, we have shown that castration for 4 weeks and flutamide, a testosterone antagonist, induce analgesia in the late phase of formalin test, which is related to increase of 5-HT levels in the dorsal horn of the lumbar spinal cord (Nayebi and Ahmadiani, 1999). More recently, we have shown that fluoxetine, a specific serotonin reuptake inhibitor, in the following acute and chronic (7 days) administration induces analgesia (Nayebi et al., 2001). This result is in agreement with another study that shows that pain produced by formalin is under the modulation of monoaminergic (noradrenergic and serotoninergic) descending inhibitory systems (Omote et al., 1998). The results obtained in the present study show that acute administration of fluoxetine significantly diminished pain-related behaviour in 4-week castrated rats, which was expressed 20 min after formalin injection and lasts for 40 min (late phase). These results are in accordance with our previous reports that suggest an antinociceptive role for castration and fluoxetine (Nayebi and Ahmadiani, 1999; Nayebi et al., 2001).

It has also been reported that plasma concentrations of testosterone decrease significantly in 2-week castrated rats (Long et al., 1983). In our present study, the analgesia induced by castration was reversed by intraperitoneal administration of testosterone enanthate. Thus, we can assume that castration-induced analgesia is related to decrease in serum testosterone levels. In addition, Flutamide also produced analgesia. This further suggests an antinociceptive effect of testosterone reduction.

The antinociceptive effect of fluoxetine observed in the present study substantiates other published reports showing that 5-HT reuptake inhibitors such as alaproclate, citalopram, clomipramine, zimelidine and fluoxetine have an analgesic effect in the formalin test (Fasmer et al., 1989; Lund et al., 1991; Smith, 1998; Sawynok et al., 1999). Other animal studies show a variety of effects on nociception; both reduced and increased responses to nociceptive stimuli, which may partly reflect differences among drugs, doses and tests (Richenberg et al., 1985). In the present study, an intraperitoneal single dose administration of fluoxetine induced an analgesic effect in the late phase. Other reports have shown that inhibition of 5-HT uptake was positively correlated with plasma concentration of fluoxetine. Therefore, we suggest that the analgesic effect

of fluoxetine is due to an increase in 5-HT levels in the CNS.

Activation of serotonergic neurons in the raphe magnus is not necessary for opioid analgesia (Gao et al., 1998), but we observed that the antinociceptive effect of intraperitoneally administered fluoxetine was reduced by naloxone pretreatment. Furthermore, the analgesic effect of flutamide was also reversed by naloxone. Our data suggest that there might be possible interaction between serotonergic and opioidergic systems. Other report shows that endogenous opiate-like substances may be involved in 5-HT-produced antinociception at the spinal level through different types of opiate receptors (Yang et al., 1994).

In the present study, chemical neurolysis of spinal 5-HT terminals by intrathecal pretreatment with 5,7-DHT decreased antinociceptive effect of intraperitoneally administered flutamide. Hence, the spinal 5-HT levels would effectively modulate the analgesic effect of flutamide. Our finding supports other data on possible interactions between testosterone and serotonergic neurons (Long et al., 1983; Cowburn and Payne, 1994; Shakir et al., 1996; Nayebi and Ahmadiani, 1999). Whether this interaction between 5-HT and flutamide in the spinal cord could explain the mechanism of flutamide analgesia at the supraspinal level requires further studies. In addition, to gain a full understanding of the effect of 5-HT on castration and flutamide-induced analgesia, experiments with selective 5-HT receptor ligands would be essential.

Decrease in serum testosterone levels or block of its receptor is the basis of the therapeutic approach in malignant prostate carcinoma. In preliminary studies, combination therapy of flutamide with finasteride, a steroid-like inhibitor of 5^{α}-reductase, has been used as an alternative treatment of prostate carcinoma, and patients taking flutamide experienced better pain relief than finasteride alone (Datta et al., 1997; Staiman and Lowe, 1997; Rosendahl et al., 1999). Since serotonin is involved in modulation of nociception and there is an interaction between testosterone and serotonergic systems, fluoxetine is considered a reasonable candidate to enhance flutamide analgesia. In conclusion, our data demonstrate that coadministration of fluoxetine with flutamide significantly increases the analgesic effect of flutamide. We offer that fluoxetine, which increases synaptic 5-HT, may allow improvement of antinociceptive effect of flutamide in prostate carcinoma, and the concurrent use of these drugs may produce good pain control at doses that should avoid the side effects associated with larger doses of each individual drug. However, the exact mechanism of testosterone interaction with serotonergic system is not clear and remains to be elucidated.

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