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Effects of orchidectomy and testosterone replacement on mouse enkephalin-degrading aminopeptidase activity in the HPA axis

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Abstract

Opiates are involved in the regulation of several functions in the hypothalamus–pituitary–adrenal (HPA) axis under physiological conditions. The aim of the present work is to study the influence of orchidectomy and testosterone (T) replacement on soluble (S) and membrane bound (MB) enkephalin-degrading aminopeptidase (EDA) activities in the HPA axis. Forty male mice (Balb/C) were distributed in five groups: sham-operated control (C), orchidectomized (OR-C), and orchidectomized treated with increasing doses of T (3, 6 or 12 mg/kg). In hypothalamus, orchidectomy did not modify either S or MB EDA, although T replacement increased S but not MB EDA. In pituitary, neither S nor MB EDA activities changed with orchidectomy, although both activities changed after T replacement. On the other hand, in adrenal glands, orchidectomy increased S and MB EDA activities, whereas T replacement returned both activities to control levels. These results suggest a direct effect of T in S and MB EDA activities and therefore, an influence on their endogenous substrates regulation.

Keywords: Opiates; Enkephalinase; Testosterone; Orchidectomy; HPA axis; Mouse

1. Introduction

Numerous studies support an important contribution of the endogenous opiate peptides system in the mediation, modulation, and regulation of the stress response through the hypothalamus–pituitary–adrenal (HPA) axis (Drolet et al., 2001). Enkephalins (ENK) have been implicated in a wide range of physiological processes, with their roles in behavior, neuroendocrinology, and pain transmission being the best documented (Herz, 1993). It has been assumed that ENK are hydrolyzed by specific enzymes, leading to their inactivation. To date, most information about ENK degradation has been described in brain tissue. Two enzymatic pathways are considered to be of great importance for the degradation of ENK (Hersh, 1982). These are the hydrolysis of the Gly–Phe bound by the membrane-bound enzyme neprilysin (Roques et al., 1993) and the breakdown of the Tyr–Gly bond by the enkephalin-degrading tyrosyl aminopeptidase (EDA) (Fernández et al., 2002). Previous results from our laboratory have suggested an influence of gonadal steroids on serum aminopeptidase activities (Martínez et al., 1997, 1998), raising the possibility that such substances help to create a biochemical environment that regulates, at least in part, the activity of these enzymes. The HPA axis is subject to gonadal influence, indicated by sex differences in basal and stress HPA function and by the neuropathologies associated with HPA dysfunction (Canny et al., 1999; Viau, 2002). It is thought that the differences observed between males and females reflect differential effects of sex steroids on the HPA axis. In fact, the activity of many proteins which regulate the axis are directly regulated by steroids, and sex steroids receptors are present at many levels of the axis (Bethea et al., 1996; Herbison, 1995; Hirst et al., 1992; Madigou et al., 1996). Particularly, testosterone modulates HPA activity in an attempt to prevent the deleterious effects of HPA activation on the reproductive function (Handa et al., 1994).

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In the present study we have measured soluble (S) and membrane-bound (MB) EDA activities in hypothalamus, pituitary, and adrenal glands, in orchidectomized male mice and after testosterone (T) replacement in order to analyze the influence of the hormonal status on EDA activity and its regulatory functions on susceptible substrates in the HPA axis.

2. Material and methods

Forty male Balb/C mice were used (28.89 ± 0.71 g body weight). The animals were randomly divided into 5 groups of 8 mice each. All the animals had free access to food and water and were housed at a constant temperature of 25 °C with lights on from 7:00 a.m. to 7:00 p.m. Four groups of mice were orchidectomized and the fifth group was sham-operated and used as control (C). As previously described (García et al., 2003; Nyby and Simon, 1987; Thurman et al., 1989), 15 days after gonadectomy, three of these orchidectomized groups were treated subcutaneously with 3, 6 or 12 mg/kg of testosterone propionate (T) (Sigma, Madrid, Spain) dissolved in sesame oil, for 10 days. The fourth orchidectomized group (OR-C) and the control were only treated with sesame oil, used as vehicle. After this time the animals were anaesthetized 24 h after the last T injection by an intraperitoneal administration of chloral hydrate. Then, hypothalami, whole pituitaries and adrenal glands were obtained. The hypothalami were dissected in agreement with the stereotaxic atlas of Paxinos and Watson (1982). The hypothalamus was considered to comprise the area between 7.7 and 3.7 mm anterior to the interaural line. The tissues were frozen at −80 °C until they were used for measurement of enzyme activity.

Tissue samples were homogenized in 10 volumes of 10 mM HCl–Tris buffer (pH 7.4) and ultracentrifuged at 100,000*g* for 30 min (4 °C) to obtain the soluble fraction. The resulting supernatants were used to measure soluble enzymatic activity and protein content, assayed in triplicate. To solubilize membrane bound proteins, the pellets were rehomogenized in HCl–Tris buffer (pH 7.4) plus 1% Triton X-100. After centrifugation (100,000*g*, 30 min, 4 °C) the supernatants were used to measure MB activity and proteins, also in triplicate. To ensure complete recovery of activity, the detergent was removed from the medium by adding adsorbent polymeric Biobeads SM-2 (100 mg/ml) (BioRad, Richmond, USA) and shaking the samples for 2 h at 4 °C. Proteins were quantified in triplicate using BSA as a standard.

EDA activity was measured in a spectrophotometric assay using L-tyrosyl-β-naphthylamide (TyrNNap) (Sigma, Madrid, Spain), in accordance with a previously described method (Ramírez-Expósito et al., 2002), with modifications. Briefly, 15 μL of each supernatant were incubated for 90 min at 37 °C with 100 μL of the substrate solution: 100 μM TyrNNap and 1.5 mM bovine serum albumin (BSA) in 50 mM of phosphate buffer, pH 7.4. The reaction was stopped by adding 100 μl of 0.1 M acetate buffer, pH 4.2, with 2% Fast Garnet. The amount of β-naphthylamine released as a result of the enzymatic activity was measured spectrophotometrically at 550 nm wavelength. Specific S and MB EDA activities were expressed as pmol of TyrNNap hydrolyzed per min per mg of protein by using a standard curve prepared with the latter compound under corresponding assay conditions. The spectrophotometric assay was linear with respect to time of hydrolysis and protein content. We used one-way analysis of variance (ANOVA) to analyze differences between groups. Post hoc comparisons were made using the least significant difference test; P values below 0.05 were considered significant.

3. Results

Specific soluble and membrane-bound EDA activities in the hypothalamus, pituitary, and adrenal gland are shown in Figs. 1–3.

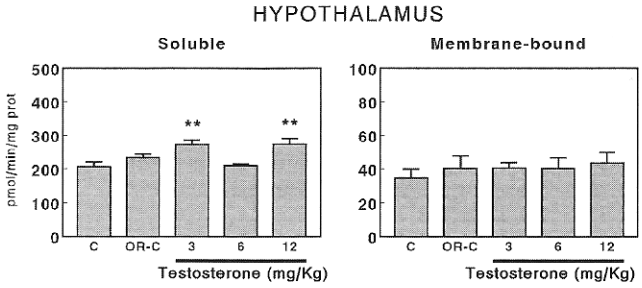

Fig. 1. Specific soluble and membrane bound enkephalin-degrading-aminopeptidase (EDA) activities in pituitary of sham-operated control (C), orchidectomized (OR-C) and orchidectomized groups administrated with 3, 6 and 12 mg/kg of testosterone. Results are expressed in picomoles of tyrosyl-β-naphthylamide hydrolyzed per min and per mg of protein (means ± SEM; $n = 8$; $^{**}P < 0.01$).

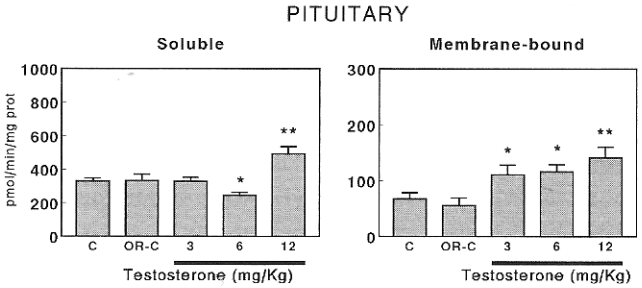

Fig. 2. Specific soluble and membrane bound enkephalin-degrading-aminopeptidase (EDA) activities in pituitary of sham-operated control (C), orchidectomized (OR-C) and orchidectomized groups administrated with 3, 6 and 12 mg/kg of testosterone. Results are expressed in picomoles of tyrosyl-β-naphthylamide hydrolyzed per min and per mg of protein (means ± SEM; $n = 8$; $^{*}P < 0.05$; $^{**}P < 0.01$).

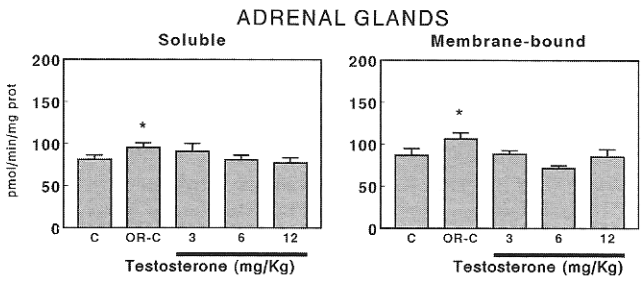

Fig. 3. Specific soluble and membrane bound enkephalin-degrading-aminopeptidase (EDA) activities in adrenal glands of sham-operated control (C), orchidectomized (OR-C) and orchidectomized groups administrated with 3, 6 and 12 mg/kg of testosterone. Results are expressed in picomoles of tyrosyl-β-naphthylamide hydrolyzed per min and per mg of protein (means ± SEM; $n = 8$; $^*P < 0.05$).

Orchidectomy did not modify S or MB EDA either on the hypothalamus or the pituitary (Figs. 1 and 2). However, in hypothalamus, the administration of 3 and 12 mg/kg T significantly increased ($P < 0.01$ in both cases) S EDA activity, whereas MB EDA activity was not modified with T replacement (Fig. 1).

In pituitary, T replacement with 6 mg/kg significantly decreased ($P < 0.05$) S EDA activity, whereas T replacement with 12 mg/kg significantly increased ($P < 0.01$) S EDA activity. In the same way, all doses of T significantly increased ($P < 0.05$, 0.05, and 0.01, respectively) MB EDA activity (Fig. 2).

In adrenal gland (Fig. 3) S EDA activity significantly increased ($P < 0.05$) with orchidectomy, while T replacement return the activity to control levels. In the same way, orchidectomy significantly increased ($P < 0.05$) MB EDA activity, whereas the administration of T return the activity to control levels (Fig. 3).

4. Discussion

Gonadal steroids are critically involved in several aspects of brain development and function, with considerable interest in their role in the control of HPA axis. Evidence accumulated over the last years demonstrates that the neuroendocrine response to stress display profound gender-specific differences, the manifestation of which largely depends on the presence of gonadal steroids (Burgess and Handa, 1992; Viau and Meaney, 1991). Gonadal hormones apparently exert "organizing" and "activating" effects on several neural mechanism that control HPA activity under basal and stress-related condition (Patchev and Almeida, 1996). Opioid peptides system participates in the mediation, modulation, and regulation of stress response through HPA axis (Drolet et al., 2001).

Met-ENK released from the adrenal medulla in response to stress, modulates the antinociception that accompanies stress. Antibodies against Met-ENK partially diminish electro acupuncture-induced analgesia at both levels of the midbrain and spinal cord (Han and Terenius, 1982). An ENK analog, FK 33-824, has potent analgesic activity and highly selective μ receptor activity against experimental pain in humans (Roby et al., 1983).

Considerable evidence has also appeared connecting the mechanism of action of some antidepressant drugs with the inhibition of the enzymes responsible of ENK degradation (Gallego et al., 1998). Some inhibitors of EDA have antidepressive effects (De Felipe et al., 1986; Tejedor-Real et al., 1995) and some antidepressant drugs increase ENK levels (De Felipe et al., 1985). In fact, it seem that the increase in ENK levels in the rat brain caused by antidepressant could be at least partly due to the decrease in their degradation. Others findings suggest that gonadal hormones may modulate opioid antinociception, and in particular estradiol may modify the magnitude of the response to drugs such as amphetamine and morphine (Negus and Mello, 2002).

We report here the influence of orchidectomy and T replacement on EDA activity levels in HPA axis in male mice. Our results show that orchidectomy does not modify EDA activity either in hypothalamus or pituitary, suggesting that their endogenous substrates are not modified. In hypothalamus, it has been described that endogenous opioid peptides and their receptors are regulated by gonadal steroid hormones in female but not male rats (Hammer et al., 1993). Moreover, slot blot hybridization analysis of RNA isolated from the hypothalamus indicates that estrogen treatment increases proenkephalin (PE) mRNA levels in ovaridectomized female rats, but had no measurable effect on PE mRNA levels in gonadectomized males. Furthermore, T treatment of gonadectomized males also had no effect on PE gene expression (Romano et al., 1990). These also agree with the observation that the enkephalinergic fiber system in much denser in the hypothalamus of females than in males, representing the anatomical substrate underlying various sexually differentiated neuroendocrine processes and behaviors (Watson et al., 1986). Furthermore, Nikolarakis et al. (1989) found that the met-ENK content of hypothalamus from 1-week castrates was not changed from control levels, although was reduced in those from 4-week castrates. Therefore, a differential response between short- and long-term effects of castration must be also considered.

In pituitary, it has been described that castration of male rats decreased both met-ENK and leu-ENK-like immunoreactivity, and the diminished levels of both peptides were partially restored by the administration of dihydrotestosterone (Yoshikawa and Hong, 1983). Therefore, our results at pituitary level do not explain these behaviors, and the influence of T administration on EDA activity remains unclear. However, it may be

possible that ENK degradation at pituitary level could be not modified by the hormonal status, but influences the level of the intracellular stores of ENK, detected by immunochemistry.

In the present report we also describe that the administration of T modifies EDA activity in hypothalamus and pituitary in different degrees, under conditions which were not altered by orchidectomy. These effects are probably due to pharmacological rather than physiological effects. However, little is known about the influence of steroid hormones on peptidase activities. We had evaluated the effect of gonadectomy and the in vitro response to the presence in the medium of steroid hormones on several peptide-regulating peptidases (Martínez et al., 1997, 1998). Aminopeptidase N and aminopeptidase B activities were measured in sera from male, female, orchidectomized, and ovaridectomized mice. Our results demonstrated highly significant sex differences, and an influence of steroid hormones on peptidase activity. Depending on the nature of the peptidase, these enzymes responded in different ways to the presence of these substances and also responded differently to gonadectomy. Furthermore, the changes observed in EDA activity in hypothalamus and pituitary could be due to we have used whole tissues, which includes several cell types that may be affected in different degree by gonadal steroids, that must be also taken into account. However, the fact that orchidectomy does not modify EDA activity either in hypothalamus or pituitary, indicates that the regulation of this enzyme activity in these locations, at least at short-term is not affected by T, although T replacement schedules could affect EDA activity at different neuroendocrine levels, probably at long-term.

On the contrary, our results show that orchidectomy increases both S and MB EDA activity in adrenal glands. In this location, T replacement returns EDA activity to control levels. There is much evidence to suggest that glucocorticoid secretion may be influenced by the innervation to the adrenal gland, and that this effect may be mediated by ENK. In fact, met-ENK causes a dose-dependent increase in corticosterone secretion (Hinson et al., 1994). The increase in EDA activity in adrenal glands induced by orchidectomy may indicate changes in the availability of its endogenous substrates, which may be responsible of the altered corticosterone release from the glands induced by androgens (McCormick et al., 2002). These altered functions are restored after T replacement.

To conclude, T influence EDA activity at different levels of the HPA axis, suggesting that the regulation of peptides such as ENK through their degrading enzymes may be modified by the hormonal status, modulating their function, which must be taken into account in studies about stress, depression, and pain.

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