



Hormonal status modifies renin-angiotensin system-regulating aminopeptidases and vasopressin-degrading activity in the hypothalamus-pituitary-adrenal axis of male mice

María Jesús García, José Manuel Martínez-Martos, María Dolores Mayas, María Pilar Carrera, María Jesús Ramírez-Expósito*

Unit of Physiology, Department of Health Sciences, Faculty of Experimental and Health Sciences, University of Jaén, Paraje "Las Lagunillas" s/n, E-23071, Jaén, Spain

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Abstract

Local renin-angiotensin systems (RAS) have been postulated in brain, pituitary and adrenal glands. These local RAS have been implicated, respectively, in the central regulation of the cardiovascular system and body water balance, the secretion of pituitary hormones and the secretion of aldosterone by adrenal glands. By other hand, it is known that the hypothalamus-pituitary-adrenal (HPA) axis is involved in blood pressure regulation, and is affected by sex hormones. The aim of the present work is to analyze the influence of testosterone on RAS-regulating aminopeptidase A, B and M activities and vasopressin-degrading activity in the HPA axis, measuring these activities in their soluble and membrane-bound forms in the hypothalamus, pituitary and adrenal glands of orchidectomized males and orchidectomized males treated subcutaneously with several doses of testosterone. The present data suggest that in male mice, testosterone influences the RAS- and vasopressin-degrading activities at all levels of the HPA axis. © 2003 Elsevier Science Inc. All rights reserved.

Keywords: Aminopeptidases; Sex; Testosterone; Renin-angiotensin system; Hypothalamus-pituitary-axis

Introduction

The renin angiotensin system (RAS) is involved in the control of blood pressure and fluid and electrolyte homeostasis. In addition to the circulating RAS, local systems have been postulated in brain,

* Corresponding author. Tel.: +34-953-002-010; fax: +34-953-012-141.

E-mail address: mramirez@ujaen.es (M.J. Ramírez-Expósito).

pituitary and adrenal glands, by immunodetection of its components in various mammalian species (Kon, 1996; Vila-Porcile and Corvol, 1998; Deschepper et al., 1986; Mulrow, 1993). These local RAS have been implicated, respectively, in the central regulation of the cardiovascular system and body water balance, the secretion of pituitary hormones (Wright et al., 1995) and the secretion of aldosterone by adrenal glands (Volpe et al., 1997). Classically, angiotensin II (Ang II) has been considered as the effector peptide of the RAS, but Ang II is not the only active peptide. Several of its degradation products, including angiotensin III (Ang III) and angiotensin IV (Ang IV) also possess biological functions. These peptides are formed via the activity of several aminopeptidases (Chansel and Ardaillou, 1998). Thus, Ang III is obtained by deletion of the N-terminal aspartic residue by glutamyl-aminopeptidase (GluAP) (EC 3.4.11.7) and aspartyl-aminopeptidase (AspAP) (3.4.11.-). AspAP and GluAP have been named together as aminopeptidase A (APA) or angiotensinase. Ang III is further converted to Ang IV by arginyl-aminopeptidase (aminopeptidase B, APB), (EC 3.4.11.6) or alanyl-aminopeptidase (aminopeptidase M, APM) (EC3.4.11.14) (Barret and Rawlings, 1998; Ward et al., 1990). Ang III possesses most of the properties of Ang II and shares the same receptors. This peptide is particularly important in brain and specially in pituitary physiology and plays a major role in the secretion of arginine-vasopressin (AVP) (Ardaillou, 1997). AVP is also implicated in the regulation of blood-pressure (Szczepanska-Sadowska, 1996) and is metabolized by vasopressin-degrading cystyl-aminopeptidase activity (AVP-DA) (EC 3.4.11.3) (Barret and Rawlings, 1998). It has been extensively reported that AP A, B, M and AVP-DA play an important role in the regulation of the activity of angiotensins and AVP.

By other hand, it is known that the hypothalamus-pituitary-adrenal (HPA) axis is involved in blood pressure regulation, and is affected by sex hormones. The aim of the present work is to analyze the influence of testosterone on RAS-regulating APA, APB and APM activities and AVP-DA activity in the HPA axis, measuring these activities in their soluble and membrane-bound forms in the hypothalamus, pituitary and adrenal glands of orchidectomized males and orchidectomized males treated subcutaneously with several doses of testosterone. The present data suggest that in male mice, testosterone influences the RAS- and AVP- regulating activities at all levels of the HPA axis.

Material and methods

Forty male BALB/C mice (body weight 26.76 ± 1.01 g) were used in this study. The animals were housed under constant temperature (25 °C) and day length (12 hours). The experimental procedures for animal use and care were in accordance with the European Community Council Directive (86/609/EEC). All animals were allowed access to water and food ad libitum. The animals were randomly distributed into five groups of eight animals each. One sham-operated group was considered as control (C) and the other four groups were orchidectomized. Fifteen days after the orchidectomy, three of these groups received a subcutaneous injection of 3, 6 and 12 mg/kg body weight of testosterone in sesame oil during 10 days, whereas one group received the vehicle only and was considered as orchidectomized control (OR-C). Sham-operated group also received the vehicle. After this time, the animals were killed under chloral hydrate anaesthesia (280 mg/Kg body weight). Hypothalamus, pituitary and adrenal glands were quickly removed and frozen at -80 °C until their use. To obtain the soluble fraction, 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). The resulting supernatants were used to measure soluble enzymatic activities and

protein content, assayed in triplicate. To solubilize membrane 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 solubilized membrane-bound activities and proteins, also in triplicate. To ensure complete recovery of activities, the detergent was removed from the medium by adding to the samples adsorbent polymeric Biobeads SM-2 (100 mg/ml) (Bio-Rad, Richmond, CA) and shaking for 2 h at 4 °C. APM, APB and AVP-DA were measured spectrophotometrically using alanyl- β -naphthylamide (AlaNNap), arginyl- β -naphthylamide (ArgNNap) or cystyl- β -naphthylamide (CysNNap) as the substrate, as previously described (Ramírez-Expósito et al., 2001). Briefly, 15 μ l of each supernatant were incubated for 90 min at 37 °C with 100 μ l of the substrate solution: 100 μ M AlaNNap, 100 μ M ArgNNap or 100 μ M CysNNap and 1.5 mM bovine serum albumin (BSA) in 50 mM of phosphate buffer, pH 7.4 for AlaNNap and ArgNNap and 50 mM Tris-HCl pH 6.0 for CysNNap. AspAP was also determined spectrophotometrically in triplicate, with aspartyl- β -naphthylamide (AspNNap) as the substrate, as previously described (Ramírez-Expósito et al., 2001). 15 μ l of each supernatant was incubated with 100 μ l of the substrate solution (100 μ M AspNNap, 1.5 mM BSA and 2 mM MnCl₂ in 50 mM HCl-Tris buffer pH 7.4) during 90 min at 37 °C. GluAP was measured as previously described (Ramírez-Expósito et al., 2001), using as substrate glutamyl- β -naphthylamide (GluNNap). 15 μ l of supernatant was incubated for 90 min at 37 °C with 100 μ l of the substrate solution (100 μ M GluNNap, 1.5 mM BSA and 50 mM CaCl₂ in 50 mM HCl-Tris buffer pH 7.4). All the reactions were stopped by adding 100 μ l of 0.1 M acetate buffer, pH 4.2 with 0.2% *Fast Garnet GBC*. The β -naphthylamine released as the result of the enzymatic activities is coupled to the GBC salt and the resultant compound was measured spectrophotometrically at 550 nm wavelength. Proteins were quantified in triplicate by the method of Bradford, using BSA as a standard. Specific soluble and membrane-bound APM, APB, APA and AVP-DA activities were expressed as pmoles of Ala-, Arg-, Asp-, Glu- and CysNNap 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 of time of hydrolysis and protein content.

Analysis of data and statistics

To analyze the influence of orchidectomy and testosterone on APM, APB, APA, and AVP-DA activities, we used an analysis of variance (ANOVA), followed by LSD post-hoc test. All comparisons with *p* values below 0.05 were considered significant.

Results

Specific soluble and membrane-bound AspAP and GluAP (APA), APM, APB and AVP-DA activities in the hypothalamus, pituitary and adrenal glands are shown in Figs. 1–5.

Regarding APA, soluble AspAP and GluAP activities were significantly higher ($p < 0.01$) in the hypothalamus after the orchidectomy. However, the administration of 3 mg/Kg of testosterone did not decrease either AspAP or GluAP activities. Only the administration of higher doses of testosterone (6 and 12 mg/Kg) returned the levels of both activities to the control values (Figs. 1 and 2). Membrane-bound AspAP and GluAP activities did not change either with orchidectomy or with the administration of testosterone (Figs. 1 and 2).

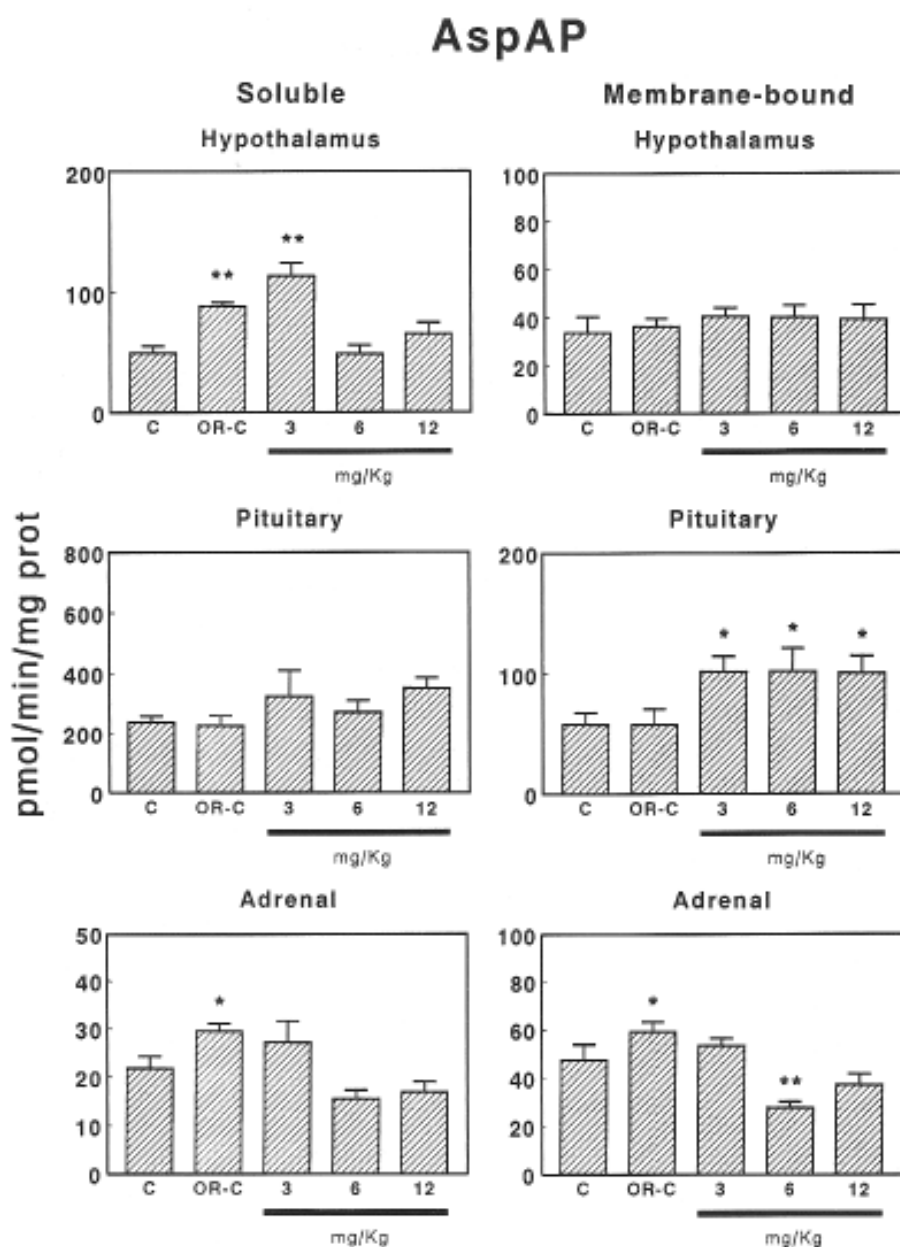


Fig. 1. Specific soluble and membrane-bound aspartyl-aminopeptidase (AspAP) activity in hypothalamus, pituitary and adrenal glands of control (C), orchidectomized (OR-C) and orchidectomized groups administrated with 3, 6 and 12 mg/Kg of testosterone. Results are expressed in picomoles of aspartyl- β -naphthylamide hydrolyzed per min and per mg of protein (Mean \pm SEM; $n = 8$; * $p < 0.05$; ** $p < 0.01$).

In pituitary, orchidectomy did not change soluble AspAP and GluAP activities. Furthermore, the administration of 3, 6 and 12 mg/Kg of testosterone did not change soluble AspAP activity, but soluble GluAP activity significantly decreased ($p < 0.05$) or increased ($p < 0.05$) with the administration of 6 and 12 mg/Kg of testosterone respectively (Figs. 1 and 2). Orchidectomy did not change membrane-bound AspAP and GluAP, but the administration of 3, 6 and 12 mg/Kg of testosterone increased significantly ($p < 0.05$) both activities (Figs. 1 and 2).

In adrenal glands, orchidectomy increased significantly ($p < 0.05$) soluble AspAP and GluAP activities, whereas the administration of testosterone returned the levels of both soluble AspAP and

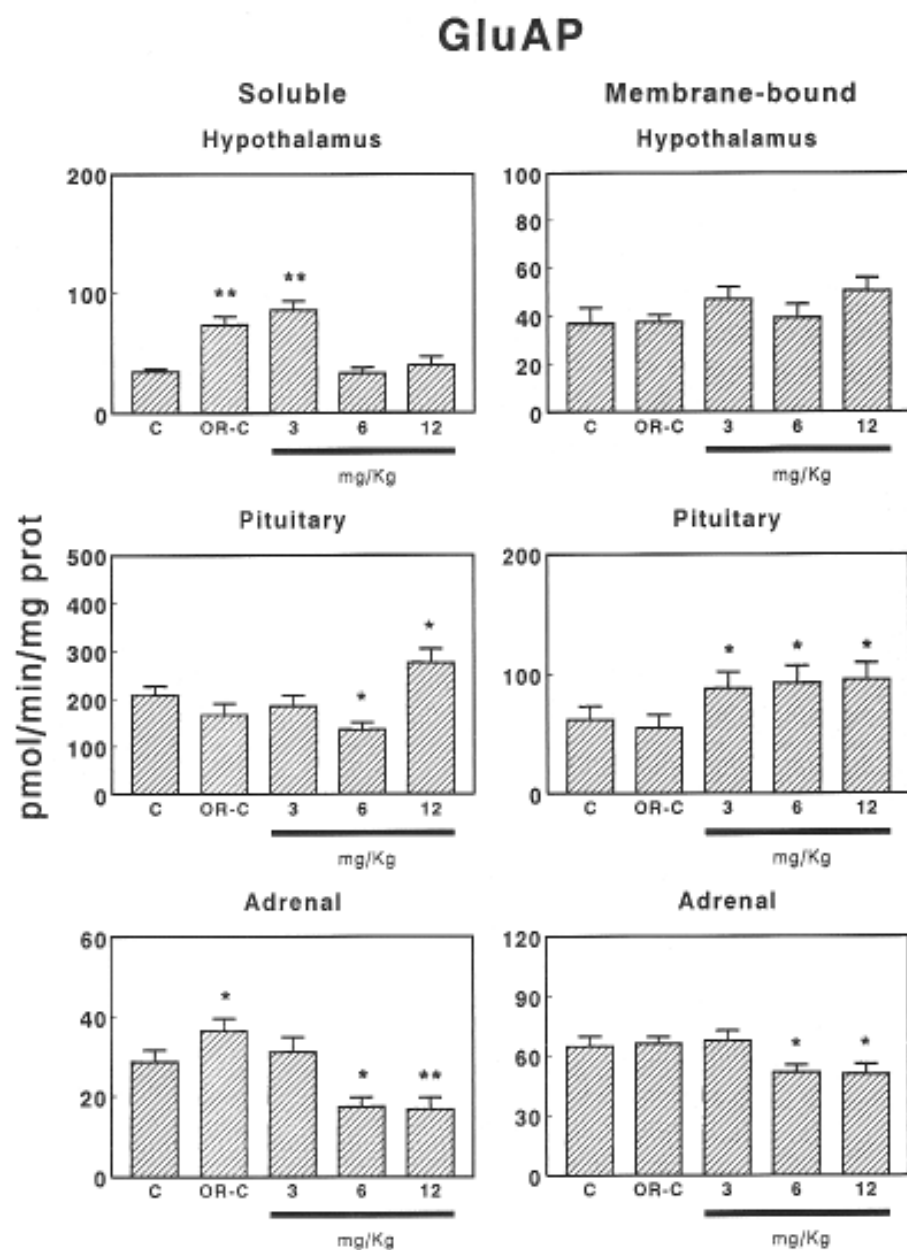


Fig. 2. Specific soluble and membrane-bound glutamyl-aminopeptidase (GluAP) activity in hypothalamus, pituitary and adrenal glands of control (C), orchidectomized (OR-C) and orchidectomized groups administrated with 3, 6 and 12 mg/Kg of testosterone. Results are expressed in picomoles of glutamyl- β -naphthylamide hydrolyzed per min and per mg of protein (Mean \pm SEM; n = 8; *p < 0.05; **p < 0.01).

GluAP activities to the control levels, although the higher doses of testosterone induced a significant decrease ($p < 0.05$ for 6 mg/Kg and $p < 0.01$ for 12 mg/Kg) on soluble GluAP activity (Figs. 1 and 2). Membrane-bound AspAP but not GluAP increased significantly ($p < 0.05$) with orchidectomy, and the administration of testosterone returned the levels of AspAP activity to the control (3 and 12 mg/Kg) or under control (6 mg/Kg; $p < 0.01$) values. However, the administration of higher doses of testosterone (6 and 12 mg/Kg) decreased significantly ($p < 0.05$) membrane-bound GluAP (Figs. 1 and 2).

Regarding APM and APB, soluble activities were significantly higher ($p < 0.01$ and $p < 0.05$ respectively) in the hypothalamus after the orchidectomy (Figs. 3 and 4). The administration of 3 mg/Kg

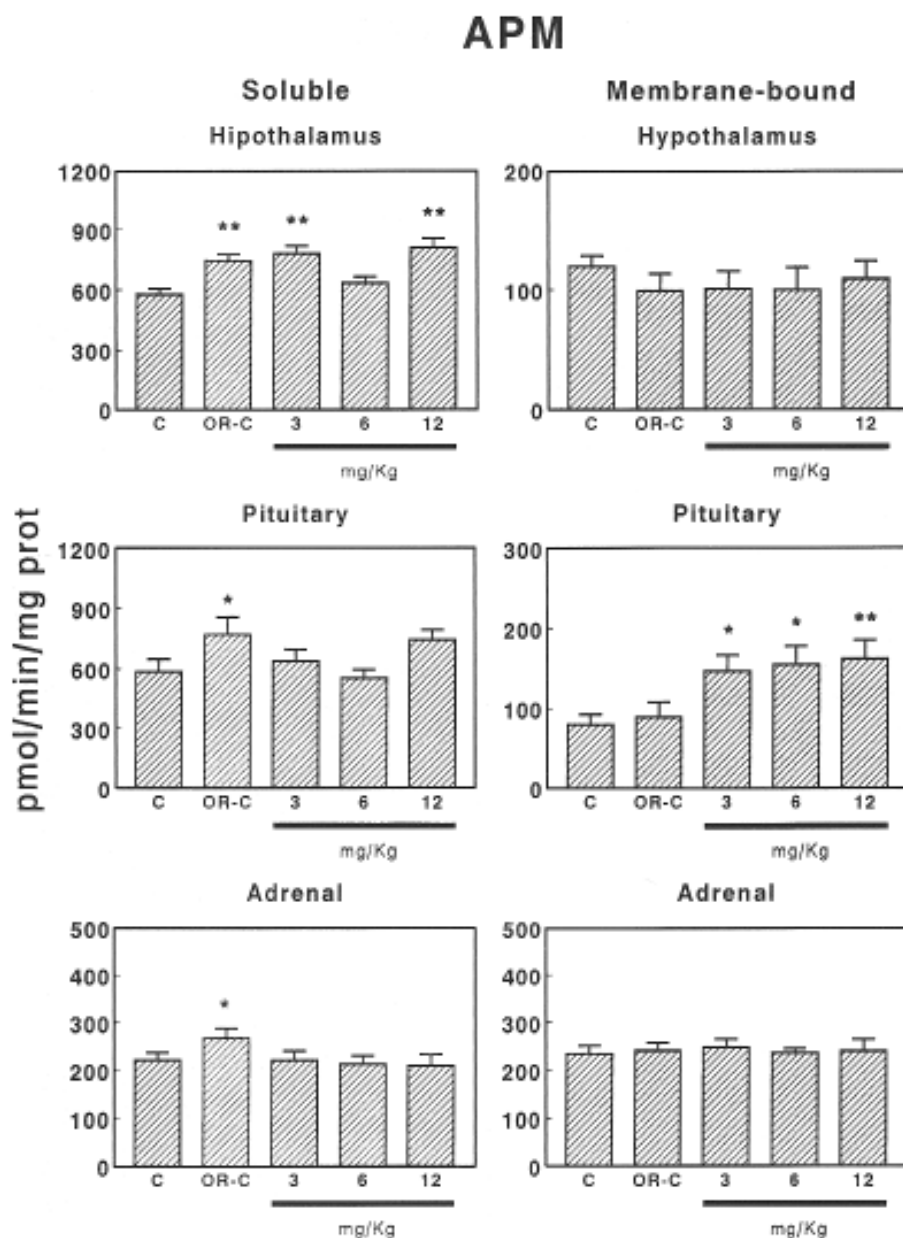


Fig. 3. Specific soluble and membrane-bound aminopeptidase M (APM) activity in hypothalamus, pituitary and adrenal glands of control (C), orchidectomized (OR-C) and orchidectomized groups administrated with 3, 6 and 12 mg/Kg of testosterone. Results are expressed in picomoles of alanyl- β -naphthylamide hydrolyzed per min and per mg of protein (Mean \pm SEM; n = 8; *p < 0.05; **p < 0.01).

of testosterone did not decrease APM or APB activities. The administration of 6 mg/Kg testosterone returned their levels to the control values. However, the administration of the higher doses of testosterone (12 mg/Kg) induced a significant increase ($p < 0.01$) in both activities (Figs. 3 and 4). Membrane-bound APM and APB activities did not change either with the orchidectomy or with the administration of testosterone (Figs. 3 and 4).

In pituitary, orchidectomy increased significantly ($p < 0.05$) soluble APM and APB activities, whereas the administration of testosterone return the levels of both soluble activities to the control levels. Moreover, 6 mg/Kg of testosterone return soluble APB activity under control levels ($p < 0.05$) (Figs. 3

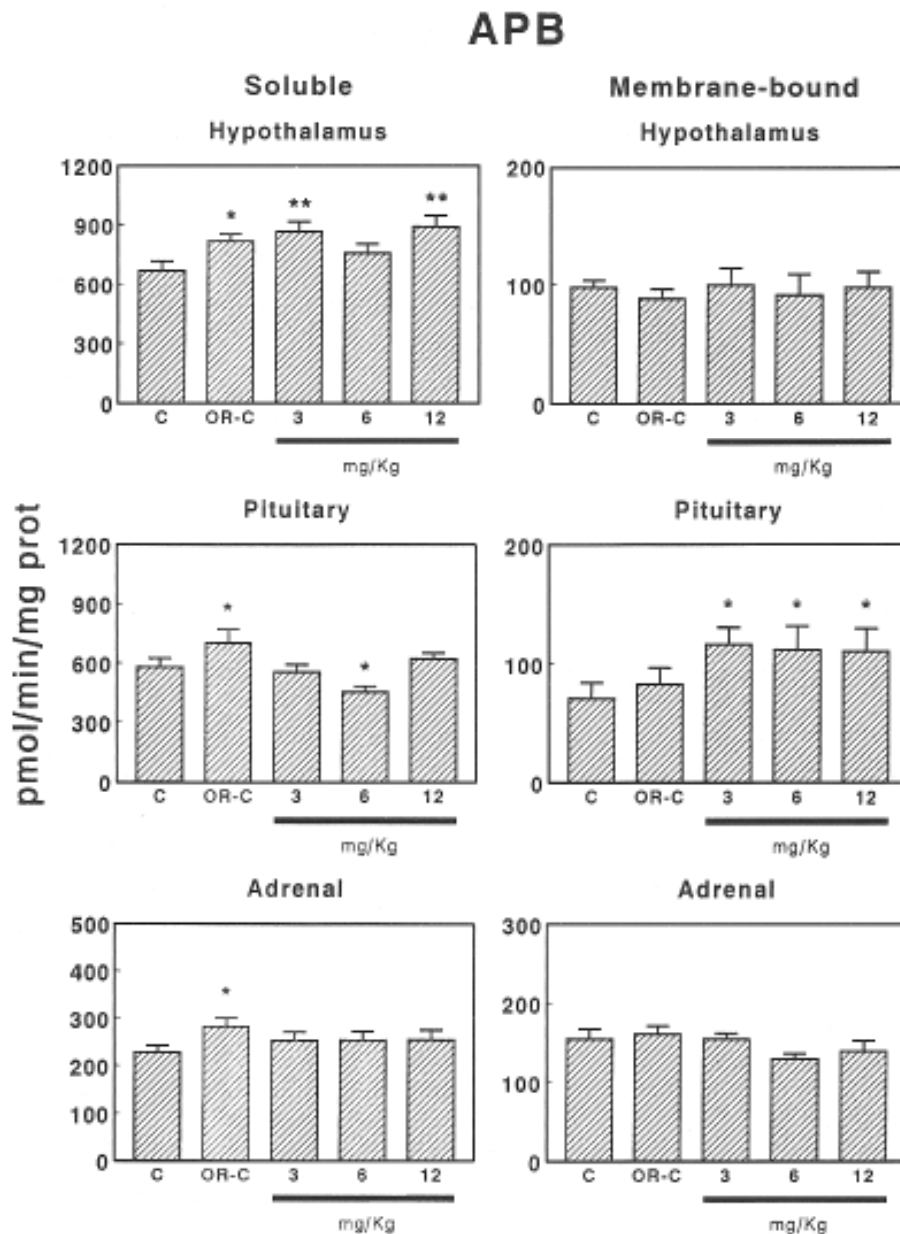


Fig. 4. Specific soluble and membrane-bound aminopeptidase B (APB) activity in hypothalamus, pituitary and adrenal glands of control (C), orchidectomized (OR-C) and orchidectomized groups administrated with 3, 6 and 12 mg/Kg of testosterone. Results are expressed in picomoles of arginyl- β -naphthylamide hydrolyzed per min and per mg of protein (Mean \pm SEM; $n = 8$; * $p < 0.05$; ** $p < 0.01$).

and 4). Orchidectomy did not change either membrane-bound APM or APB, but the administration of 3, 6 and 12 mg/Kg testosterone increased significantly ($p < 0.05$) both activities (Figs. 3 and 4).

In adrenal glands, orchidectomy also increased significantly ($p < 0.05$) soluble APM and APB activities, but the administration of any dose of testosterone returned both soluble activities to the control levels (Figs. 3 and 4). Membrane-bound APM and APB activities did not change neither with the orchidectomy nor with the administration of testosterone (Figs. 3 and 4).

Regarding AVP-DA, soluble activity did not change with orchidectomy in hypothalamus, but the administration of 6 mg/Kg testosterone decreased significantly ($p < 0.05$) this activity (Fig. 5).

AVP-DA

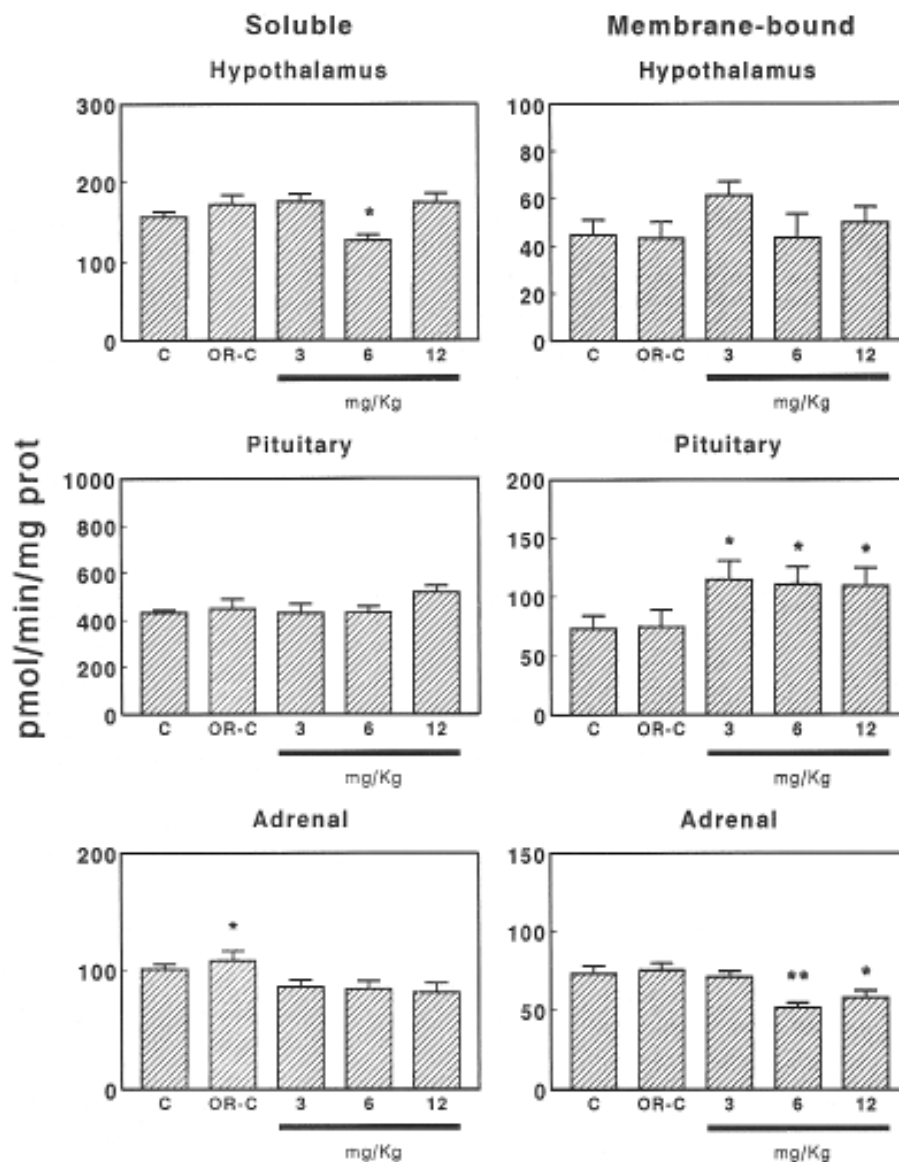


Fig. 5. Specific soluble and membrane-bound vasopressin-degrading activity (AVP-DA) in hypothalamus, pituitary and adrenal glands of control (C), orchidectomized (OR-C) and orchidectomized groups administrated with 3, 6 and 12 mg/Kg of testosterone. Results are expressed in picomoles of cystyl- β -naphthylamide hydrolyzed per min and per mg of protein (Mean \pm SEM; $n = 8$; * $p < 0.05$; ** $p < 0.01$).

Membrane-bound AVP-DA did not change either with the orchidectomy or with the administration of testosterone (Fig. 5).

In pituitary, orchidectomy did not change soluble AVP-DA either with the orchidectomy or with the administration of testosterone (Fig. 5). Membrane-bound AVP-DA did not change with orchidectomy, but the administration of 3, 6 and 12 mg/Kg testosterone increased significantly ($p < 0.05$) this activity.

In adrenal glands, soluble AVP-DA increased significantly ($p < 0.05$) after orchidectomy, whereas the administration of any dose of testosterone returned the levels to the control values (Fig. 5). Membrane-bound AVP-DA did not change with the orchidectomy, but the administration of higher doses

of testosterone induced a significant decrease ($p < 0.01$ for 6 mg/Kg and $p < 0.05$ for 12 mg/Kg) on membrane-bound AVP-DA.

Discussion

The maintenance of cardiovascular and body fluid homeostasis requires mechanisms that regulate the total amount of water and sodium in the body and their appropriate regional and compartmental distribution. Most of these mechanisms are mediated through HPA axis. The recognition that HPA axis hyperactivity increases blood pressure is only over the last decade that evidence has emerged to support the concept. Thus, the action of hypothalamic secretagogues, most notable corticotropin-releasing factor (CRF) and AVP from the paraventricular nucleus, results in a marked increase in the release of adrenocorticotropin (ACTH) from the anterior pituitary (Whitnall, 1993). ACTH in turn causes the release of glucocorticoids (and probably aldosterone (Lumbers, 1999)) from the adrenal cortex into circulation, increasing blood pressure (Kelly et al., 1998). In this way, it has been described that the hypertensive effects of ACTH infusion are reproduced by intravenous or oral glucocorticoid administration in a dose-dependent fashion (Kelly et al., 1998, 2001; Whitworth et al., 1997, 2000). By other hand, it is well established that sex steroids have several effects in the HPA axis, and that these effects occur at virtually all levels of the axis. Thus, it has been described effects in the levels of type I and II glucocorticoid receptors in the hippocampus and hypothalamus (Turner and Weaver, 1985; Carey et al., 1995; MacLusky et al., 1996), the expression of CRF and AVP in the hypothalamus (Vanvakopoulos and Crousos, 1993; Paulmyer-Lacroix et al., 1996; Viau and Meaney, 1996), the secretion of ACTH from the anterior pituitary (Gallucci et al., 1993) and the size and steroidogenic potential of the adrenal glands (Kitay, 1961; Gaskin and Kitay, 1970; Roelfsma et al., 1993; El-Migdadi et al., 1995). It is though that these effects of sex steroids on the HPA axis occur modifying the activity of many proteins which regulate the axis at their different levels (Hirst et al., 1992; Herbison, 1995; Bethea et al., 1996; Madigou et al., 1996).

These effects of sex steroids on HPA axis may explain gender differences in blood pressure described in human and rat models of hypertension. In rat models, males have higher blood pressures than do females (Masubuchi et al., 1982; Rowland and Fregly, 1992; Chen and Meng, 1991; Ashton and Balment, 1991). For example, the male spontaneous hypertensive rat (SHR) has higher blood pressures than does the female SHR at a similar age (Masubuchi et al., 1982; Kumai, 1991). Recent studies using the technique of 24-hours ambulatory blood pressure monitoring have also confirmed in humans that blood pressure is higher in men than in premenopausal women at similar ages (Wiinberg et al., 1995; Khoury et al., 1992; Reckelhoff et al., 1998). In fact, although the mechanism(s) responsible for higher blood pressures in male versus females is not clear, in rats androgens have been shown to promote hypertension, in view of the fact that castration of male SHR decreases blood pressure and chronic testosterone treatment of ovariectomized female SHR increases blood pressure (Masubuchi et al., 1982). Ovariectomy alone does not affect hypertension in females, suggesting that it is not estrogen that protects female SHR from the development of hypertension but a lack of androgens. In human, the link between androgens and blood pressure has been further strengthened by studies using ambulatory blood pressure monitoring in children; these studies have shown that boys have higher blood pressures than do age-matched girls and that there is a significant greater increase in blood pressure at puberty in males than in females (Ouchi et al., 1987; Bachmann et al., 1987).

In animal models, it has been found that the elevated blood pressure found in all genders of SHR is reduced by enalapril treatment, implicating the RAS in the development of hypertension of all SHR regardless of gender. However, and most important it has been also found that the higher blood pressures in male SHR and ovariectomized female SHR treated with testosterone were normalized by chronic enalapril treatment, further implicating the RAS in androgen-mediated hypertension (Reckelhoff et al., 1999). It has been also shown that renal angiotensinogen mRNA levels in normotensive rats are affected by gender, with angiotensinogen mRNA being higher in kidneys from males than from females (Guyton et al., 1972; Kaufman et al., 1998). Castration decreases and testosterone treatment of ovariectomized females increases angiotensinogen mRNA (Guyton et al., 1972; Kaufman et al., 1998). Estrogen treatment of ovariectomized females rats has also been shown to reduce angiotensin-converting enzyme activity and mRNA (Hall et al., 1990). In human, other studies have also shown that sex hormones affect the RAS; thus, plasma renin activity is higher in men than in aged-matched women (Reckelhoff et al., 1997; Ely et al., 1991). It has also been shown that plasma renin activity is higher in postmenopausal women than in premenopausal women or postmenopausal women receiving hormone replacement therapy (Marks et al., 1999).

To our knowledge, the present work describes for the first time the influence of sex steroids on RAS-regulating APA, APB and APM activities and AVP-DA in male mice HPA axis. Thus, orchidectomy modifies hypothalamus APA, APM and APB; pituitary APM and APB; and adrenal APA, APM and APB and AVP-DA, whereas testosterone replacement return the activity to control or under control levels, depending on the dose used. We also describe that the administration of the highest doses of testosterone modifies some activities which were not altered by orchidectomy, probably due to pharmacological rather than physiological effects. This presumable pharmacological effects also appears when the highest doses of testosterone are used under conditions in which lower doses return the activity to control levels after being modified by orchidectomy. However, little is known about the influence of steroid hormones on AP activities. We have evaluated the effect of gonadectomy and the *in vitro* response to the presence in the medium of steroid hormones. APN and APB activities were measured in sera from male, female, orchidectomized and ovariectomized mice. Our results demonstrated highly significant sex differences, and an influence of steroid hormones on AP activity. Depending on the nature of the AP, these enzymes responded in different ways to the presence of these substances and also responded differently to gonadectomy (Martínez et al., 1998). Therefore, further research is necessary to fully understand this topic.

Regarding to the different levels of the HPA axis, it is well known that several hypothalamic nuclei are involved in neuroendocrine regulation through RAS action. Thus, *in vivo* administration of AngII appears to act by stimulating CRF secretion (Ganong and Murakami, 1987). Furthermore, AngIII behaves as one of the main effector peptide of the RAS in the control of AVP release (Zini et al., 1996, 1998). In fact, AngIII possesses most of the properties of AngII and may also activate AT1 and AT2 receptors (Lenkey et al., 1997; Ardaillou, 1997). Our data may indicate that, under androgen deprivation, AngII is converted into AngIII by APA more rapidly in hypothalamus, being AngIII also quickly converted into AngIV by APB and APM. Although AngIV has been implicated mainly in memory and cognitive processes and sensory and motor integration (Wright et al., 1995), it has been also described that AngIV produces opposing responses to AngII with respect to local tissue blood flow, regulating in certain degree, AngII/AngIII functions (Swanson et al., 1992). Therefore, the functions of AngII and AngIII may be diminished and blunt the release of CRF and AVP from hypothalamus. In fact, long-term orchidectomy decreased hypothalamic CRF content (Haas and George, 1988). However, several studies

suggest that the effects of androgen in the CNS to augment HPA axis activity are in part secondary to an interaction with corticosteroid negative feedback mechanisms. In males, orchidectomy increased ACTH responses whereas androgen treatment decreased ACTH response (Handa et al., 1994). Testosterone replacement, however, appeared to decrease AVP content in the median eminence (Viau and Meaney, 1996), and more recent studies suggest that the primary effect of testosterone is to decrease AVP mRNA, rather than CRF mRNA (Viau et al., 1999). However, the effect on AVP mRNA was observed as an interaction with adrenal steroids (Viau et al., 1999). Taken together, the results suggest that androgens interact with adrenal steroids in regulation of AVP synthesis in the hypothalamus. These results also suggest that the site of steroid hormone action is the hypothalamus rather than the pituitary.

In the pituitary RAS, AngII stimulates in a paracrine manner the secretion of adrenocorticotrophin (ACTH) (Ganong, 1989). Our data may indicate that androgen deprivation does not change APA activity, but induces the conversion of Ang III to Ang IV more rapidly, maintaining the levels of Ang II, which could be also regulated by AngIV, helping to maintain low levels of ACTH. In this way, Lesniewska et al. (1990) have described that orchidectomy decreases pituitary ACTH levels in rats. These data may indicate that low levels of glucocorticoids will be present in castrated males.

In adrenal glands, there is little doubt that both circulating and intra-adrenal RAS can stimulate aldosterone secretion, being AngII the principle regulator of its secretion in the adult animal (Lumbers, 1999). Our data show the same pattern of RAS-regulating aminopeptidases than in hypothalamus. Thus, AngII may be converted into AngIII by APA more rapidly and then quickly converted into AngIV by APB and APM, which could be responsible of low aldosterone levels. In any case, there may be other functions of the intra-adrenal RAS apart from these direct effects on aldosterone production, involved in the local regulation of adrenal blood flow, which must be also taken into account.

We can conclude that the increasing effects of testosterone in blood pressure may be mediated by changes in the RAS-regulating aminopeptidase activities at different levels of the HPA-axis and AVP-DA in adrenal glands.

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