Assessment of brain AT1-receptor on the nocturnal basal and angiotensin-induced thirst and sodium appetite in ovariectomised rats

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Abstract

Objective. Considering the controversial data regarding the role of the brain renin-angiotensin system (RAS) on the thirst and sodium appetite in ovariectomised rats, we aimed to evaluate the role of the brain angiotensin II (Ang II) AT1-receptor on the nocturnal fluids intake.

Materials and methods. Groups of Wistar female rats were ovariectomised and chronically given oestrogen or vehicle to evaluate its influence on effects induced by i.c.v. injection of losartan, Ang I and Ang II.

Results. The i.c.v. losartan decreased basal water intake in the ovariectomised group. Ang I but not Ang I-induced nocturnal dipsogenic and natriorexigenic responses in ovariectomised rats. In oestrogen-treated rats, both peptides increased fluids intake. Previously, i.c.v. losartan abolished these effects in all groups. Oestrogen replacement decreased the nocturnal fluids intake, attenuated the losartan and Ang II effects, and highlighted the Ang I response.

Conclusions. The present study has shown for the first time the involvement of AT1 receptor in regulating nocturnal basal water and salt intake in ovariectomised rats. In addition, our data have revealed an unexpected increased brain Ang I-mediated fluid intake in oestrogen-treated ovariectomised rats, which was blocked by previous i.c.v. losartan. Our data have therefore shown that oestrogen influences homeostatic behaviours dependent on brain RAS.

Introduction

It is widely recognised that the influence of the peripheral and brain renin-angiotensin system (RAS) is an essential constituent of the mechanism mediating water and electrolyte balance with regard to sodium appetite and thirst response. The central angiotensin II (Ang II) AT1-receptors were identified along the lamina terminalis, in the circumventricular organs (CVO's) especially in the subfornical organ (SFO) organum vasculosum laminae terminalis (OVLT) and neighbouring circuits inside the blood–brain barrier. Those loci have been implicated in the mediation of the dipsogenic and natriorexigenic actions of Ang II elicited by hypovolaemic and/or hyponatraemic stimuli and also in the basal condition. Recently, Sakai's group has successfully obtained strong genetic evidence implicating de novo synthesis of Ang II in the SFO as an integral player in fluid homeostasis. In addition to the AT1-receptors identified into the SFO, this new evidence strengthens the concept since all components of RAS are found in it.

It has been shown that the involvement of ovarian steroids in fluid and electrolyte balance is mostly through actions on the RAS. One of these studies demonstrated that Ang II AT1-receptor binding increased in ovariectomised rats and decreased in oestrogen replacement ovariectomised rats (EB-OVX) in the hypothalamus and especially in SFO. Moreover, Kisley and colleagues have shown that oestrogen administered to OVX rats increased Ang II-induced c-Fos labelling in the lateral magnocellular neurons of the paraventricular hypothalamic nucleus (PVN).

These data above show that fluid and electrolyte metabolism could be disrupted in OVX rats and therefore, their homeostatic behavioural responses would be modified by sodium and volume alteration challenges. Tanaka's group has demonstrated that oestrogen therapy attenuates the drinking response induced by Ang II activation of the specific SFO projections to the median preoptic nucleus (MnPO). Likewise, there is evidence that oestrogen replacement decreases the dipsogenic response induced by activation of angiotensinergic pathways, which project from the lateral hypothalamic area to the SFO.

Regarding sodium appetite, Kensicki and colleagues have demonstrated that increasing plasma oestrogen levels by systemic administration was positively correlated with daily sodium intake in OVX rats. Moreover, Ang II microinjected into the MnPO induced salt intake in OVX of lesser magnitude compared to intact female rats. The same group had also observed that oestrogen therapy decreased the water intake and abolished the saline ingestion induced by intracerebroventricular (i.c.v.) injection of Ang II in OVX rats.
Chow and colleagues reported no changes in daily sodium consumption in adult OVX rats. However, Stricker’s group has shown an increased appetite for salt in OVX rats after prolonged sodium deprivation. Once more, the salt appetite response was blunted by oestrogen therapy. It has also been demonstrated that the inhibitory role of oestrogen on salt appetite in rats appears to occur only when the appetite is especially pronounced under natriorexigenic challenge.

Considering the controversial data reported herein, we aimed to evaluate the thirst and sodium appetite responses in adult OVX and EB-OVX rats with i.c.v. administration of losartan, an AT\textsubscript{1}-receptor antagonist. Finally, we have intended to investigate the effect of AT\textsubscript{1}-receptor blockade on the fluid intake response attained by i.c.v. administration of Ang I and Ang II.

**Material and methods**

**Animals, maintenance and drugs**

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and pertinent Brazilian legislations. It has been approved by the institutional animal ethics and welfare committee.

Sixty-days-old Wistar female rats were bilaterally ovariectomised and kept in metabolic cages. They received fluids offered in graduated bottles in a room with light on from 7:00 a.m. to 7:00 p.m., under controlled temperature (24±2˚C) and ad libitum access to Purina food pellets and fluids. Distilled water and hypertonic saline (1.8% NaCl, solution normally aversive for gustatory perception in rats) were used to evaluate the dipsogenic and sodium appetite responses during the experimental challenges. At the end of the experiments, the animals were sacrificed with a high dose of sodium thiopental.

The neurotransmitters/peptide hormones, Ang I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and the AT\textsubscript{1}-receptor antagonist, losartan (108 nmol/animal) solutions in a final 2-μl volume over a period of 30 seconds, using a 2-μl Hamilton syringe connected to a stainless-steel obturator. On the end of stereotaxic surgery a stainless-steel obturator was put into the cannula. On the day of the experiment, the obturators were removed and the animals were given i.c.v. injection of saline, Ang I (77 pmol/animal), Ang II (96 pmol/animal) or losartan (108 nmol/animal) solutions in a final 2-μl volume over a period of 30 seconds, using a 2-μl Hamilton syringe connected to a stainless-steel injector by polyethylene tubing. Before experiments the animals were adapted to the metabolic cages where all experiments were carried out.

At the beginning of the experiments the body weight of rats varied slightly (200 and 220 g) in both groups. In order to validate the ovariectomy and oestrogen replacement, two weeks after surgery representative OVX and EB-OVX rats (n=18/group) were sacrificed and their uteri were removed to determine the uterine index, expressed as uterine weight (mg) divided by 100 g of body weight. The data showed a significant difference in uterine index of the OVX compared to EB-OVX (53.5±6.5 mg/100 g vs. 353.0±28.5 mg/100 g body weight, p<0.001, by unpaired Student t-test) which ascertained the procedures efficacy. All surgeries were made under anaesthesia induced by ketamine (60 mg/kg, i.p.) and xylazine (7.5 mg/kg, i.p.) and were given a prophylactic dose of veterinary antibiotics (Fort Dodge, Brazil).

**Intracebroventricular administration**

For i.c.v. administrations, guide stainless-steel cannulae (26-gauge, o.d. 0.6 mm, length 14 mm) were stereotactically implanted into the anterior ventral portion of the 3rd ventricle (AP = -0.5 to -0.4 mm posterior to bregma; L = 0 mm; V = 8.3 to 8.5 mm below the skull calvaria) as described previously according to the coordinates of Paxinos and Watson atlas. The upper part of the cannula was attached to the skull with methyl methacrylate and fixed on the bone surface with stainless-steel screws. The location of the cannula was confirmed by cerebrospinal fluid reflux and routine procedures. The animals were allowed to recover for 5–7 days. During this period they were handled daily and maintained in individual cages. At the end of stereotaxic surgery a stainless-steel obturator was put into the cannula. On the experiment day, the obturators were removed and the animals were given i.c.v. injection of saline, Ang I (77 pmol/animal), Ang II (96 pmol/animal) or losartan (108 nmol/animal) solutions in a final 2-μl volume over a period of 30 seconds, using a 2-μl Hamilton syringe connected to a stainless-steel injector by polyethylene tubing. Before experiments the animals were adapted to the metabolic cages where all experiments were carried out.
Experimental procedures
In order to observe the rats during the activity period, we performed all experimental sessions between 7:00 p.m. to 1:00 a.m., when visceral sensory activity (from the gustatory, olfactory and volume receptors) and hedonic drives seems to be somewhat increased in rats. This schedule allows examination of salt intake in the basal situation and, further, to investigate the intensity of dipsogenic and sodium appetite responses during the nocturnal period.

Four series of experiments were carried out (n=8 to 12, each group) in OVX and EB-OVX rats as follows:

**Effect of oral administration of losartan on the Ang II-mediated fluid intake**
OVX and EB-OVX rats received oral treatment with losartan or distilled water during five weeks. Thereafter, the rats were randomly assigned to the following groups. I – Control group: rats treated solely with i.c.v. saline; II – Ang II group: rats treated solely with i.c.v. Ang II; III – LOS group: rats treated with losartan in the drinking water plus i.c.v. saline; IV – LOS + Ang II group: rats treated with losartan in the drinking water plus i.c.v. Ang II. Both OVX and EB-OVX rats underwent identical I to IV protocols. This protocol was designed to investigate the influence of chronic oral losartan treatment on the water and salt intake induced by i.c.v. Ang II injection. Following oral administration, losartan and its metabolite EXP3174 permeate the blood-brain barrier and access neuronal AT\(_1\)-receptors.27

**Acute effect of i.c.v. losartan injection on fluid intake**
OVX and EB treated-OVX rats underwent i.c.v saline (saline group) or LOS injection (LOS group) in order to investigate the role of AT\(_1\)-receptor on the nocturnal basal thirst and sodium appetite.

**Acute effect of i.c.v. losartan injection on Ang I-mediated fluid intake**
OVX and EB treated-OVX rats were randomly assigned to receive losartan or saline i.c.v. followed by saline (saline + saline group) or Ang I (saline + Ang I and LOS + Ang I groups) injection 60 minutes later. This design allowed us to evaluate the influence of Ang I i.c.v. administration and further, investigate the effect of previous AT\(_1\)-receptor blockade on the Ang I-induced nocturnal fluid intake.

**Acute effect of i.c.v. losartan injection on Ang II-mediated fluid intake**
In order to evaluate the effect of AT\(_1\)-receptor blockage on Ang II-mediated response, we performed the same design as described above. In this case, the experimental groups were formed as follows: saline + saline, saline + Ang II and LOS + Ang II groups.

**Statistical analysis**
Data were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni test. Unpaired “t” Student test was used when two groups were compared. The analyses were performed by using the statistical GraphPad Prism software (version 4, Inc., San Diego, USA). In all comparisons, the level of significance was set at p<0.05.

**Results**
Chronic oral treatment with losartan increases salt intake but does not alter Ang II-mediated responses in ovariectomised rats
As expected, i.c.v. administration of Ang II increased water intake in OVX and EB-OVX groups compared with their controls (figure 1a). Chronic losartan treatment neither altered basal water intake nor i.c.v. Ang II administration showed a tendency to increase the salt intake

[Graph and Table Descriptions]
consumption (p<0.09 and p<0.06, respectively, figure 1b), independently of the previous treatment with losartan.

**AT1-receptor is important in mediating basal water intake only in ovariectomised rats**

Nocturnal i.c.v. injection of losartan decreased basal water intake in O VX rats as shown in figure 2a. This effect was not observed in O VX-replaced rats probably as a result of the low basal level in the EB-O VX group. Regarding salt intake, EB O VX-replaced rats showed a decrease in this parameter, compared to O VX non-replaced rats. This effect seems not to be mediated by AT1-receptor, inasmuch as losartan treatment did not change this response pattern in both groups (figure 2b).

**Oestrogen treatment highlights water and salt intake following Ang I i.c.v. injection**

Although central Ang I administration did not alter basal nocturnal water intake, but this parameter was significantly decreased when O VX rats were previously treated with losartan by i.c.v. route (figure 3a). This effect resembles that observed in those O VX rats solely treated with i.c.v. losartan (figure 2a). Interestingly, the EB replacement seems to restore the Ang I-mediated water intake response in O VX-replaced rats, which have a low basal water intake level as shown in figures 2a and 3a. Once again, this effect was decreased with previous administration of losartan. Despite of salt intake, Ang I i.c.v. injection increased this response only in EB-O VX rats that was normalised by previously central losartan administration.

**Oestrogen treatment attenuates water and salt intake induced by Ang II i.c.v. injection**

Ang II induced a marked dipsogenic response that was strongly inhibited by prior central losartan treatment in O VX rats. Although Ang II also induced an increase in water intake in EB-O VX rats, this response was attenuated. Losartan was also effective in blocking this response (figure 4a). This response was suppressed by previous losartan i.c.v. injection. Like water intake, i.c.v. Ang II administration induced an increased salt intake in both groups, but it was highlighted in O VX rats. It also was inhibited by previous losartan injection (figure 4b). Altogether, these data suggest that EB may modulate basal and Ang I and II-mediated fluid ingestive behaviour.

**Discussion**

The present study was encouraged by controversial data regarding dipsogenic and natriorexigenic responses in O VX rats and the oestrogenic influence on these behaviours. As far as we know, we have described for the first time a high brain angiotensinergic sensitivity suggested by an increase in basal and Ang II-mediated fluid intake in O VX non-replaced rats.

Chronic oral losartan administration during five weeks significantly increased salt intake response in O VX non-replaced compared to O VX replaced rats. This behavioural evidence is supported by findings regarding chronic losartan-induced hypotension and renal sodium loss. Thornton and colleagues have hypothesised that the mechanism underlying salt intake elicited by chronic hypotension may involve direct barosensitive input to the sodium appetite centres of the brain. Since oestrogen administration reduces RAS activity, it is plausible to accept the concept by which oestrogen-deficient rats express higher brain sensitivity to AT1-receptor blockade than the treated rats.

In line with the fact that Dean and colleagues have demonstrated an increase in brain angiotensin-converting enzyme (ACE) activity and binding densities of the AT1-receptor in O VX rats, it is reasonable that losartan-induced
hypotension and sodium urinary loss could be enhanced in these animals compared to OVX-replaced rats. Therefore, our data show that oestrogen-treatment may prevent the increase in salt intake response through chronic AT1-receptor blockade in OVX rats.

Interestingly, chronic oral treatment with losartan did not influence the nocturnal basal water intake or even the dipsogenic effect induced by acute Ang II i.c.v. administration in OVX rats. EB therapy also did not alter Ang II action as well. These data suggest that long-term oral losartan treatment did not change the specifically dipsogenic response at least in our protocol. The long-term decrease in water intake evoked by losartan i.c.v. injection alone seems to strongly indicate an AT1-receptor mediated component in the nocturnal basal dipsogenic response. This observation contrasts with that described by Reis and colleagues, which demonstrated no difference when losartan was injected into the lateral ventricle in normo-hydrated rats. However, those results were achieved in different conditions, such as employment of male intact rats and the schedule of diurnal administration of losartan.

Here, we have provided evidence that EB therapy significantly decreases nocturnal basal water intake. This observation is comparable to that of Findlay and colleagues, who have shown that spontaneous drinking was less during pro-oestrus and oestrus. Furthermore, these authors showed that oestrogen-treated OVX rats exhibited a decreased spontaneous drinking response. Losartan centrally administered did not influence the nocturnal basal salt intake response in either OVX replaced and non-replaced rats. At present, we have no explanation for this, however, we could postulate that oestrogen treatment modifies differentially the AT1-receptors densities or AT1-receptors-activation ratio to

Figure 3
Influence of previous i.c.v. losartan (108 nmol/animal) administration on fluids intake induced by i.c.v. Ang I (77 pmol/animal) in OVX and EB-OVX groups: Water (a) and salt (b) intake two hours after i.c.v. Ang I administration. Values are expressed as means±SEM from 10–12 animals. * = p<0.05 vs. corresponding control group. † = p<0.05 vs. corresponding no i.c.v. losartan injection (one-way ANOVA). OVX = vehicle-treated ovariectomised; EB-OVX = oestrogen-treated ovariectomised.

Figure 4
Influence of previous i.c.v. losartan (108 nmol/animal) administration on fluids intake induced by i.c.v. Ang II (96 pmol/animal) in OVX and EB-OVX groups: Water (a) and salt intake (b) two hours after the i.c.v. Ang II administration. Values are expressed as means±SEM from eight to ten animals. * = p<0.05 vs. corresponding control group. † = p<0.05 vs. corresponding no i.c.v. losartan injection (one-way ANOVA). OVX = vehicle-treated ovariectomised; EB-OVX = oestrogen-treated ovariectomised.
natriorexigenic and dipsogenic responses in basal condition. Future studies based on molecular techniques will be necessary to confirm this hypothesis.

Nocturnal Ang II, but not Ang I, i.c.v. administration increased water intake response in OVX rats. Although nocturnal Ang I i.c.v. administration induced no significant dipsogenic response (compared to saline + saline i.c.v. injection) prior losartan i.c.v. injection significantly reduced the basal water intake in OVX rats.

OVX rats exhibited an intense dipsogenic response provoked by Ang II i.c.v. injection, which was powerfully depressed by prior losartan central administration. These data and others described elsewhere supported that AT1-receptor activation take part in the Ang II-mediated dipsogenic action and nocturnal basal water intake.2,20 Oestrogen treatment significantly attenuated the Ang II dipsogenic response. This reinforces the hypothesis that angiotensinergic components are down-regulated by oestrogenic influence. Present results are analogous to those in which angiotensin-induced drinking and pressure responses in females were attenuated by central EB treatment.2 In contrast, this observation differs in relation to that reported by De-Vale and colleagues which observed no difference in the Ang II-mediated dipsogenic response in OVX vs. OVX oestrogen-treated rats.2 These data have shown the controversial effect from oestrogen treatment concerning Ang II dipselective action. Our observation agrees with the reports of Fujisawa and Tanaka17,18. Both these groups have demonstrated that Ang II dipsogenic action are dependent on angiotensinergic circuits between lateral hypothalamus area and SFO and SFO and median preoptic nucleus, respectively, was decreased by oestrogen replacement.17,18 Taken together, most evidence suggests that oestrogen acting centrally down-regulates angiotensinergic drives implicated in the AT1-receptor-activated dipsogenic response.

Nocturnal Ang I i.c.v. injection evoked dipsogenic responses in oestrogen-treated rats, which was decreased by previous losartan central administration. These data raise various interpretations. Oestrogen therapy could be facilitating the brain Ang I to Ang II conversion despite attenuated nocturnal basal and Ang II-mediated water intake. This hypothesis does not corroborate evidence previously reported, which showed that ACE activity in the brain had been prevented by regular estradiol replacement and reversed by high estradiol treatment in OVX rats.32 Consistent data from Dean and colleagues support the evidences that Ang II AT1-receptor binding is increased in OVX rats and oestrogen replacement decreases this response in the hypothalamus and especially in the SFO.19,34 In addition, Sakai's group have produced strong genetic evidence implicating de novo synthesis of Ang II in the SFO as an integral player in fluid homeostasis.35 Another peptide, Ang 1-7, may mediate water and salt intake evoked by Ang I i.c.v. injection. Indeed, Mahon and colleagues have found no changes on the drinking response challenged by Ang I-7 i.c.v. injection.36 However, these authors used male rats in their study, which could lead to misleading results. Thus, future studies in OVX rats are needed to clarify the actual role of oestrogen replacement at different time points as well as to investigate the brain RAS and other peptides and kinin systems.

Taking into account that losartan inhibits Ang I-mediated water intake we can suggest that this response depends upon AT1-receptor inside the blood-brain barrier.

Notwithstanding salt intake behaviour, we also observed down-regulation-like responses of the AT1-receptor in OVX rats undergoing oestrogen replacement. Indeed, Ang II-induced salt intake was significantly decreased in oestrogen-treated rats as earlier demonstrated.1,20 Curiously, as observed in water intake, Ang I i.c.v. administration stimulated the sodium appetite in oestrogen-treated rats but not in OVX oil-treated rats.

Both Ang I and Ang II-stimulated sodium appetite were similarly attenuated by prior injection of AT1-receptor antagonist in replaced rats. Although RAS was probably down-regulated, the AT1-receptor may still be essential in that behavioural response in these animals.

In conclusion, our results have shown that AT1-receptor-mediated water and salt intake responses were enhanced in OVX rats, which were prevented or partially attenuated by oestrogen replacement. As far as we know, we have described for the first time the involvement of the brain AT1-receptor in the nocturnal basal fluid intake in ovariectomised rats. In addition, our experiments have disclosed an unexpected brain Ang I-mediated fluid intake in oestrogen-treated OVX rats that was effectively blocked by previous AT1-antagonist i.c.v. administration.

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